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In order to determine whether differences exist in expression of estrogen receptor (ER) variant mRNAs between normal and tumor breast tissues, we have analyzed by reverse transcription-polymerase chain reaction (RT-PCR) ER variants mRNA expression in matched normal breast tissue and breast tumor components from 11 different patients. We have set up two assays (Triple-Primer-PCR and long-range PCR) that allow the quantitative evaluation of clone 4 ER variant and exon-deleted ER variant mRNAs expression within each samples. A general trend toward a higher expression of exon 5-deleted, exon 7-deleted ER variant and clone 4 estrogen receptor (ER) variant in the tumor component compared to the normal counterpart of matched samples has been seen. This is consistent with previous observations made on independent samples. The number of cases together with the amount of starting material (frozen tissue sections per samples) needs to be increased in order to establish confidently any statistically significant differences. The relative spatial localization of normal and tumor components together with the phenotypic characteristics of the tumors (ER and progesterone receptor values, as measured by ligand binding assay) are two parameters that will be taken into account in the selection of additional cases.

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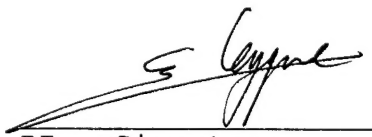

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INTRODUCTION

Beside genetic and environmental factors, hormonal factors are suspected to take part in the genesis of human breast cancer. The balance between the mitogenic action of estradiol and the antiproliferative action of progesterone is indeed thought to be directly involved in the mechanisms underlying breast tumor growth and progression.[1] These two ovarian steroids were believed, until recently, to act mainly through an estrogen (ER) and two isoforms of the progesterone (PR) receptor, respectively. The discovery in 1996 of a novel estrogen receptor referred to as ER-beta (ERβ) effectively added to the complexity of estrogen mechanisms of action.[2,3] ER and PR belong to the steroid/thyroid/retinoic receptor gene superfamily and are intracellular ligand-activated transcription factors.[4,5,6] These two receptors, that can be divided into several structural and functional domains, are encoded by transcripts containing 8 exons.

Using different techniques such as reverse transcription followed by polymerase chain reaction (RT-PCR) or Northern blot analysis, many laboratories have detected variant ER transcripts in breast cancer biopsy samples as well as normal reduction mammoplasty specimen.[7,8,9,10,11] Three main kinds of variant ER mRNAs have been identified so far in human breast tissues:

- exon-deleted variants (missing one or several exons).
- exon-duplicated (containing exon-repetition) or inserted variants (with additive sequences).
- truncated variants containing only the 5'-terminal extremity of the wild-type (WT-ER) mRNA.

Most of the predicted ER-like proteins encoded by these variants lack some of the WT-ER structural domains. For example, the putative ER-like protein encoded by an exon 5-deleted ER variant mRNA lacks most of the hormone binding domain. The putative ER-like protein encoded by an exon 7-deleted ER variant mRNA lacks the dimerization domain and much of the hormone binding domain of WT-ER, whereas the putative protein encoded by clone 4 truncated ER variant mRNA[12] (consisting of exon 1 and exon 2 of WT-ER mRNA joined to sequences unrelated to WT-ER) contains only the AF-1 region and the first zinc finger of WT-ER (see reference 11 for a review).

While it is still unclear if all these variant ER mRNAs are stably translated in vivo, putative encoded proteins are suspected to exhibit altered functions which could interfer with WT-ER signalling pathway. Functional analysis of *ex vivo* activity of some of these ER-like proteins confirmed the validity of this hypothesis. For example, in a recombinant yeast expression model, the ER-like protein encoded by the exon 5-deleted ER variant mRNA was shown to constitutively activate an estrogen responsive reporter gene[9] whereas protein encoded by the exon 7-deleted ER variant mRNA was shown to inhibit WT-ER activity.[10]

It has therefore been speculated that these variants could be involved in the acquisition of hormone independence that occurs during breast tumor progression. This hypothesis is supported by *in vivo* observations. Exon 5-deleted ER mRNA expression relative to WT-ER is effectively higher in ER-/PR+ than in ER+/PR+ tumors[9,13] whereas exon 7-deleted mRNA expression is higher in ER+/PR- than in

ER+/PR+[14] tumors. Similarly, relatively higher levels of the clone 4 truncated variant ER mRNA were found in tumors with markers of poor prognosis and lack of hormone sensitivity (PR-) compared to those with markers of good prognosis and hormone sensitivity.[15]

We have recently demonstrated that expression of exon 5-deleted variant mRNA and possibly the exon 7-deleted variant mRNA, both determined relative to WT-ER, was higher in cancer than in normal breast tissue.[16] Similarly, we have established that clone 4 mRNA expression relative to WT was significantly increased in a group of breast tumors (all ER+/PR+) compared to unmatched normal reduction mammoplasty samples.[17] Such data suggest that the molecular mechanisms generating ER variant mRNAs could be deregulated in breast cancer tissues compared to normal breast tissues, and may contribute to early steps in breast tumorigenesis.

The goal of this project is to address the possible role of Estrogen Receptor variants in human breast tumorigenesis.

Objectives:

1. To look for differences in the expression of Exon 5-deleted, exon 7-deleted and clone 4 variant mRNAs between matched normal breast tissue, invasive primary carcinoma, and metastatic carcinoma in axillary lymph nodes.

2. To identify variant ER mRNAs differentially expressed in normal breast and breast cancer tissue.

3. To determine the putative function of differentially expressed variants.

To avoid individual differences this study was planned to be performed on normal breast tissue, invasive primary carcinoma, and metastatic carcinoma in axillary lymph nodes samples coming from the same patient. All specimens are provided by the Manitoba Breast Tumor Bank.

BODY

Objective 1

Exon 5-deleted and exon 7-deleted ER variant mRNAs relative expression.

In the Manitoba Tumor Data Bank files, eleven patients have been identified from which matched normal breast tissue and primary invasive carcinoma were available. Among these cases, 6 have ER levels lower than 10 fmol/mg prot and 5 have ER values higher than 10 fmol/mg prot, as determined by ligand binding assay. Detection, analysis and quantitation of exon 5-deleted and exon 7-deleted mRNAs was performed by RT-PCR as previously described.[16] Briefly, for each patient, total RNA was extracted from the normal and tumor components of 20 μm frozen cryostat sections. Reverse transcription of total RNA using random hexamers was followed by PCR amplification using appropriate primer sets and dCTP [α-^{32}P]. PCR

products were separated on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with an intensifying screen. Quantification of signals was carried out after excision of the bands corresponding to variant and WT mRNA (using autoradiographs as a guide), followed by addition of 5 ml scintillant (ICN Pharmaceuticals, Inc, Irvine, California) and counting in a scintillation counter (Beckman). The exon-deleted signal was expressed as a percentage of the WT-ER signal.

Results obtained so far are summarized in figure 1 (exon 5-deleted ER variant) and figure 2 (exon 7-deleted ER variant).

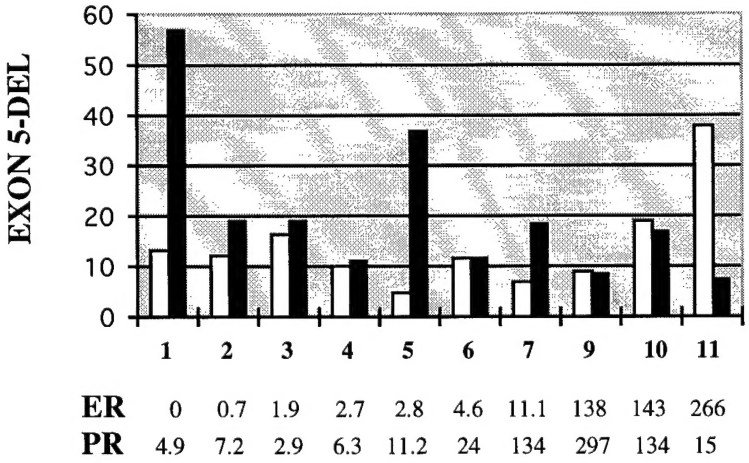

Figure 1

For each patient (1-11) exon 5-deleted signal has been expressed as a percentage of WT-ER for both normal (white) and primary invasive lesion (black). ER and PR levels of the tumor, as determined by ligand binding assay, are also indicated.

Among the eleven patients studied, one did not express a detectable level of exon-5 deleted ER variant mRNA in its normal compartment. This probably results from a generally lower level of RNA in this particular sample and therefore a lower level of ER-like RNA substrate. This problem, that we also met for other variants and using other techniques (c.f. long range RT-PCR approach, objective 2) is consistent with the much lower cellularity of normal component compared to the tumor component, especially in samples from older women. This can be solved by analyzing more cDNA, i.e more frozen sections of the corresponding case. In this first series of experiments only 10 patients have therefore been included in the comparative study. The general trend of a lower exon 5-deleted expression in normal than in the tumor component is however confirmed: the average value for the 10 cases being 14.0 % for normal versus 20.5 % for tumors, although the difference did not reach statistical significance. Out of these 10 cases, 7 expressed a higher level of exon 5-deleted variant in their tumor component than in the normal counterpart. While there tended to be a correlation between high relative exon 5-deleted variant ER mRNA expression and ER values, as measured by ligand binding assay in the tumor, the numbers of samples are too small at

this stage for any meaningful conclusions to be made. We have collected and will continue to collect more matched samples to be analyzed.

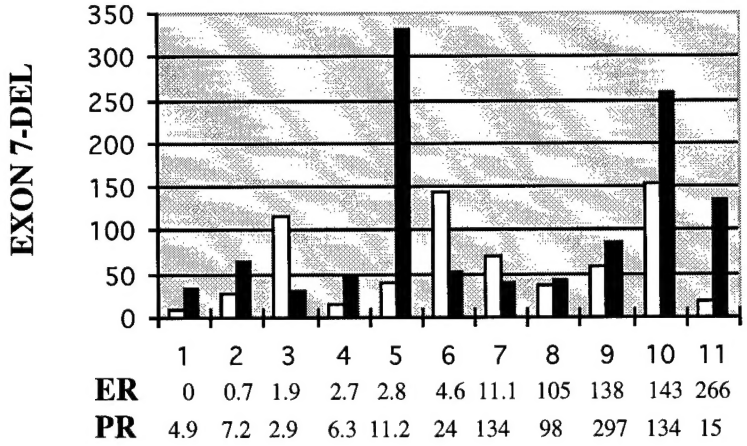

Figure 2

For each patient (1-11) exon 7-deleted signal has been expressed as a percentage of WT-ER for both normal (white) and primary invasive lesion (black). ER and PR levels of the tumor, as determined by ligand binding assay, are also indicated.

The exon 7-deleted ER variant mRNA was detected in all normal and tumor components. This is consistent with the general observation that this variant is the most highly expressed in breast tissues, particularly compared to exon 5-deleted ER variant.[18] As previously observed in independent normal and tumor breast specimens,[16] a trend to a general lower expression of this variant in normal than in tumor is also observed here: the average relative expression being 62.9 % and 101.8 % for normal and tumor samples, respectively. Out of the eleven cases studied, 8 expressed a higher level of exon 7-deleted ER variant in their tumor compartment. No obvious relationship can be found between ER or PR status, as determined by ligand binding assay. An increased number of matched samples will be analyzed for exon 7-deleted ER variant mRNA expression as well. Since the award of this grant, new data has been published highlighting the differences between morphologically "normal" tissue adjacent to a tumor component versus "real" normal tissue geographically isolated from the tumor component.[19] This is a variable in our study which we will have to now consider in our further analysis.

Clone 4 ER variant mRNA relative expression.

Due to some technical problems not previously encountered we have redesigned our triple-primer polymerase chain reaction (TP-PCR) assay that we had previously set up to quantify clone 4 variant ER mRNA expression.[17] This new TP-PCR, that uses new primers, has been validated by comparing the results obtained using that approach to those obtained using a standardized RNase protection assay. These data have been submitted for publication (see appendix 1).[20] We showed that this approach is reliable and

highly specific, and can be used to address the question of the expression of clone 4 variant mRNA relative expression in ER negative samples or samples presenting a very low ER, by binding assay.

This TP-PCR has now been applied to the study of clone 4 relative expression in normal and tumor matched samples corresponding to the eleven cases selected previously. Results obtained to date are summarized in figure 3.

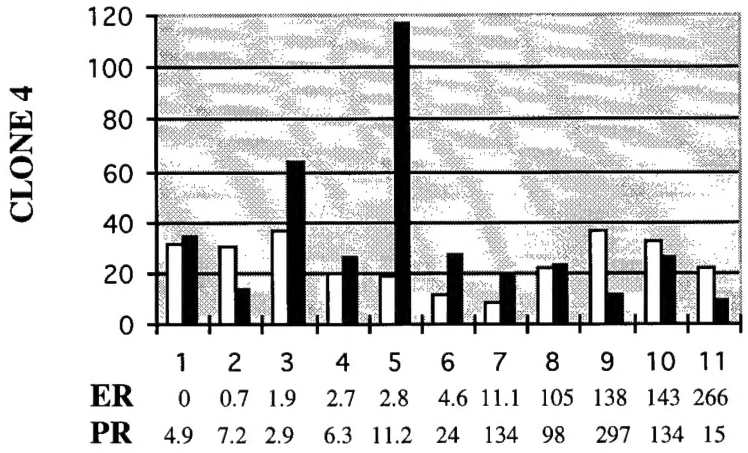

Figure 3

For each patient (1-11) clone 4 variant mRNA signal obtained after TP-PCR has been expressed as a percentage of WT-ER for both normal (white) and primary invasive lesion (black). ER and PR levels of the tumor, as determined by ligand binding assay, are also indicated.

A general trend with an higher expression in tumor than normal is again observed in these samples: averages are 24.5 % and 33.8 % for normal and tumor compartment, respectively. In 7 cases, the expression of clone ER variant mRNA relative to WT-ER is higher in tumor compartment compared to corresponding normal one. This result is consistent with previous ones obtained on unmatched normal and tumor samples.[17] Again an increased number of samples is required to confirm or refute this observation statistically.

Objective 2

Using targeted PCR, all ER variants previously identified in breast tumors were detected in normal breast tissue (ie exon 2-, exon 3-, exon 4-, exon 5-, exon 7-deleted and clone 4 truncated). This suggested that multiple ER variant mRNAs are expressed both in normal and tumor breast tissues. The pattern of expression (qualitatively and quantitatively) of the total ER variant mRNA population in breast tissues cannot be assessed using previously published techniques.

Effectively, previous analyses of ER variant mRNAs expression, including our own, have depended largely on assays that focus on limited regions of the transcripts. This in most cases will only allow the detection of one modification in any one ER-like mRNA. It is now clear that more than one modification can occur in variant transcripts.[21] Thus signals attributed to the exon 7-deleted ER variant mRNA detected by reverse transcription and polymerase chain reaction (PCR) using primers in exon 5 and 8[22] or by RNAse protection assays with probes covering the exon 6-8 junction,[23] will also include contributions from a variant deleted in both exon 4 and 7, recently identified by Madsen et al.[21] Nevertheless, these molecules may result in quite different proteins with possible differential effects on the WT-ER signalling pathway.

Since multiple variant ER mRNAs are expressed it became evident that there was a need to investigate qualitatively and quantitatively the representation of total ER variant mRNAs within any one given sample, in order to define differences that could be potentially important in vivo either as prognostic markers or as possible contributors to tumor progression. I have therefore developed a strategy to allow the investigation of known and unknown exon-deleted or inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants amongst themselves and with respect to WT-ER transcript. The approach developed, referred to as long range PCR, has been recently published (see appendice 2).[18] Briefly, cDNAs corresponding to all exon-deleted ER variants can be amplified together with the WT-ER mRNA using primers annealing with exon 1 and exon 8 sequences. We assumed and validated that a competitive amplification could therefore occur amongst all exon-deleted or inserted ER variant transcripts, that would depend on their initial relative representation; the detection of bands corresponding to specific ER variants reflecting the relative balance between ER variant mRNA species within the sample. This approach has been tested in a pilot study to determine the incidence of ER variants in a set of 100 breast tumors that were selected to represent a wide range of breast cancers with respect to ER and PR levels, size, grade and axillary nodal status.

In that study we concluded that for each sample it was possible to evaluate the proportion of each variant relative to others. By comparison between samples, it was possible to establish the differential pattern of expression of variant ER mRNAs. It should be remembered however that the truncated clone 4 like ER variants are not measured in this assay.

In order to determine whether the detection of particular variant mRNAs by long range PCR could correlate with the presence of putative ER variant proteins we have applied this approach to investigate ER variants expression within breast tumors previously assessed by immunocytochemistry. In this previous study, Huang et al.[24] showed that ER signal/status assessed immunohistochemically can be significantly different between amino-terminal and carboxy-terminal-targeted antibodies. Some breast tumor samples (consistent tumors) showed similar signals using both antibodies, while the majority of inconsistent tumors showed a higher signal using an N-terminal antibody than using a C-terminal antibody. Since many of the variant ER mRNAs are predicted to encode C-terminally truncated proteins, a possible explanation was that the inconsistent tumors express more truncated ER variant proteins than consistent tumors. This hypothesis

was addressed and the results have been published recently (see appendix 3).[25] Briefly, we showed in that study that ER variants encoding non truncated ER like proteins were detected using long range RT-PCR at the same frequency in both consistent and inconsistent tumors whereas ER variants encoding truncated ER like proteins were preferentially detected in the inconsistent tumors. The results of this study are consistent with the hypothesis that ER variant mRNAs are stably translated *in vivo* and the detection of variant ER mRNAs by long range PCR can be correlated to discrepancies observed by immunocytochemistry. It should be noted that detection of clone 4 variant mRNA by TP-PCR was also correlated to such discrepancies. More recently, an approach similar to the long range RT-PCR was described as to quantitatively assess ER variant mRNAs expression.[26] Long range PCR analyses are being tested on the matched normal and tumor samples previously selected.

CONCLUSION

A general trend toward a higher expression of exon 5-deleted, exon 7-deleted ER variant and clone 4 ER variant in the tumor component compared to the normal counterpart of matched samples has been seen in the first set of samples analyzed. This is consistent with the observations made on independent samples.[16,17] The number of cases together with the amount of starting material (frozen tissue sections per samples) needs to be increased in order to establish confidently any statistically significant differences. The relative spatial localization of normal and tumor components together with the phenotypic characteristics of the tumors (ER and PR values, as measured by ligand binding assay) are two parameters that will be taken into account in the selection of additional cases. We have set up two assays (TP-PCR[20] and long range PCR[18,25]) that allow the quantitative evaluation of clone 4 ER variant and exon-deleted ER variant mRNAs expression within each samples. Because of the recent description of a new form of estrogen receptor (ER-β)[2,3] it became compelling to investigate its presence in human breast tissue. This study (appendix 4)[27] has been conducted by RT-PCR. We showed that the expression of ER-β in breast tumor tissues was not correlated with that of ER-α, and that both ER-α positive and ER-α negative cell lines expressed ER-β mRNA. Some breast tumors and breast cell lines coexpress ER-α and ER-β mRNAs. Our preliminary data also show the presence of ER-β mRNA in normal human breast tissue. The biological significance of the presence of this new receptor in breast tissue, in particular its role in estrogen/antiestrogen action, remains to be determined. Depending on the answer to that important question, expression of ER-β within normal and tumor tissues could be investigated.

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[CANCER RESEARCH 56, 4324–4327, October 1, 1996]

Advances in Brief

Prevalence of Estrogen Receptor Variant Messenger RNAs in Human Breast Cancer[1]

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Abstract

A new approach, based on the competitive amplification of wild-type and exon-deleted estrogen receptor (ER) variant cDNAs, was used to screen 100 human breast tumors for the presence of ER variants. Already described exon 4-deleted ER mRNA was preferentially detected in tumors with lower grades ($P < 0.05$) or higher progesterone receptor levels ($P < 0.01$), whereas new ER variants, deleted in exons 2–4 or in regions within exons 3–7 were associated with higher grades ($P < 0.025$) and higher ERs ($P < 0.001$). This approach allows investigation of the expression of multiple ER variant mRNAs and may implicate them as new prognostic markers and as possible contributors to tumor progression.

Introduction

Several ER[3] variant mRNAs have now been detected in both normal and cancerous breast tissues (1–11). Although it is unclear if any or all of these mRNAs are translated *in vivo*, some of the predicted ER-like proteins, lacking some functional domains (12) of the WT-ER (Fig. 1), exhibit altered functions *in vitro*. Exon 3- and exon 7-deleted variants may act as dominant negative regulators of WT-ERs (3, 6), whereas exon 5-deleted ER has ligand-independent transcriptional activity (4, 13). Changes in the balance between ER-like molecules could be involved in perturbation of the ER signaling pathway and tumor progression (14–20). Many laboratories have begun to investigate the association between the expression of individual ER variant mRNAs and the loss of hormone-dependent growth (16, 19). However, it is now apparent that several different types of variant ER transcripts and therefore predicted proteins can be expressed together (8, 9), and the validity of investigating individual variants in isolation can be questioned. Furthermore, previous analyses have depended largely on assays that focus on limited regions of the transcript and that would be unlikely to detect more than one modification per individual variant mRNA. However, it is now clear that more than one modification can occur in variant transcripts (17). Thus, signals attributed to the exon 7-deleted ER variant mRNA detected with reverse transcription-PCR using primers in exons 5 and 8 (9) or with RNase protection assays with probes covering the exon 6–8 junction (20) may also include contributions from a variant deleted in both exons 4 and 7 recently identified by Madsen *et al.* (17). Nevertheless, these molecules may result in quite different proteins which differ in activity and modulate differentially the ER signaling pathway. Moreover, because of the lack of an approach to investigate qualitatively and quantitatively the representation of total ER variant mRNAs within any one given sample, it becomes difficult to evaluate those variants potentially important *in vivo* either as prognostic markers or as possible contributors to tumor progression. The purpose of this study was to develop a strategy that would allow the investigation of known and unknown exon-deleted or -inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants among themselves and with respect to the WT-ER transcript. The approach used is depicted in Fig. 1. cDNAs corresponding to all exon-deleted ER variants identified to date can be amplified along with the WT-ER mRNA using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences. We assumed that a competitive amplification could therefore occur among all exon-deleted or -inserted ER variant transcripts that would depend on their initial relative representation, the detection of bands corresponding to specific ER variants reflecting the balance between ER variant mRNA species within the sample. Since it is likely that alterations in the coding sequences could be translated into ER-like proteins with altered functions, we have for practical reasons confined our approach to the coding region only. This approach was tested in this pilot study to determine the incidence of ER variants in a set of 100 breast tumors that were selected to represent a wide range of breast cancers with respect to ER and PR levels, size, grade, and axillary nodal status.

Materials and Methods

Human Breast Tissues and Cell Line. All human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank. Tumors (100 cases) were chosen to represent a variety of tumor characteristics represented in the breast tumor population collected in the Manitoba Breast Tumor Bank. Thirty tumors were ER negative (ER < 3 fmol/mg protein), with PR values ranging from 0 to 25 fmol/mg protein, as measured using the ligand-binding assay. Seventy tumors were ER positive (ER ranging from 3.6 to 386 fmol/mg protein), with PR values ranging from 0 to 297 fmol/mg protein. These tumors also spanned a wide range of grades (from 4 to 9), determined using the Nottingham grading system (21), size (ranging from 1 to 6.3 cm), and nodal status (absence or presence of axillary nodes). T-47D-5 cells, which are known to express different ER variant mRNAs (11, 18), were kindly provided by Dr. R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia). Total RNA was extracted and reverse transcribed in a final volume of 15 μl as described previously (11).

Primers and PCR Conditions. The primers used consisted of 1/8U primer (5′-TGCCCTACTACCTGGAGAACG-3′, sense; located in WT-ER exon 1; 615–637) and 1/8L primer (5′-GCCTCCCCCGTGATGTAA-3′, antisense; located in WT-ER exon 8; 1995–1978). Nucleotide positions given correspond to published sequences of ER cDNA (22). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, 1 μl of reverse transcriptase mixture was amplified in a final volume of 10 μl in the presence of 10 nM [α-^{32}P] dCTP, 4 ng/μl of each primer, and 1 unit

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[3] The abbreviations used are: ER, estrogen receptor; WT, wild type; PR, progesterone receptor; D2-,D3-, D4-, D5-, D7-ER, variant mRNA deleted in exons 2, 3, 4, 5, and 7, respectively; D3-4-, D2-3-ER, variant mRNA deleted in both exons 3 and 4 and in exons 2 and 3, respectively; D4/7-ER, variant mRNA deleted in both exons 4 and 7; D2-3/7-ER, variant mRNA deleted in exons 2, 3, and 7; D2-3-4-ER, variant mRNA deleted in exons 2, 3, and 4; D-3-7-ER, variant mRNA deleted in sequences within exon 3 to within exon 7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

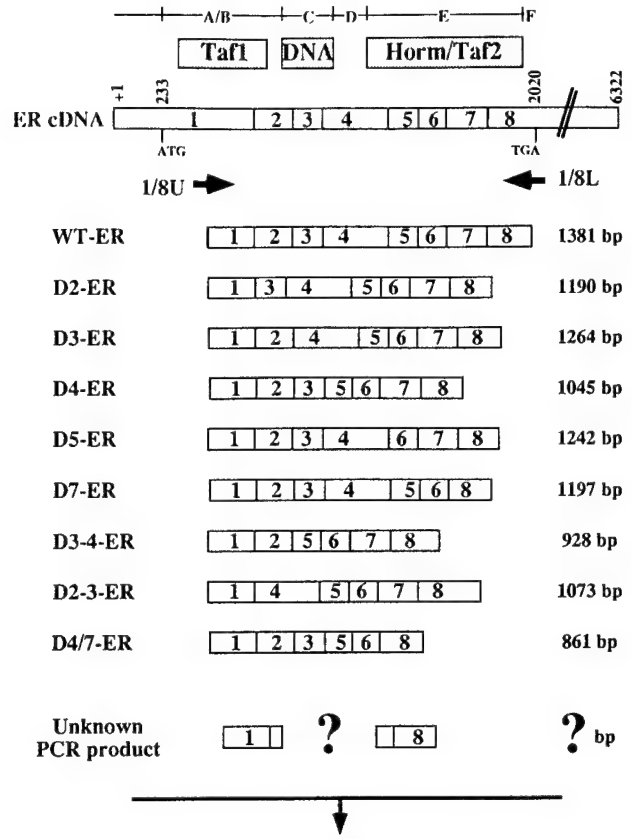

Fig. 1. Schematic representation of WT-ER cDNA and primers allowing coamplification of most of the described exon-deleted ER variants: ER cDNA contains eight different exons coding for a protein divided into structural and functional domains (*A–F*). *Region A/B* of the receptor is implicated in *trans*-activating function (*Taf1*). The DNA-binding domain is located in the *C region*. *Region E* is implicated in hormone binding and another *trans*-activating function (*Taf2*). 1/8U and 1/8L primers allow amplification of the 1381-bp fragment corresponding to WT-ER mRNA. Coamplification of all possible exon-deleted or -inserted variants which contain exon 1 and 8 sequences can occur. Amplification of the previously described ER variant mRNAs deleted in exon 2 (D2-ER), exon 3 (D3-ER), exon 4 (D4-ER), exon 5 (D5-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER), exons 4 and 7 (D4/7-ER) would generate 1190-bp, 1264-bp, 1045-bp, 1242-bp, 1197-bp, 928-bp, 1073-bp, and 861-bp fragments, respectively.

of *Taq* DNA polymerase. Each PCR consisted of 40 cycles (1 min at 60°C, 2 min at 72°C, and 1 min at 94°C). PCR products were then separated on 3.5% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. To control for errors in the input of cDNA used in PCR reactions, amplification of the ubiquitous GAPDH cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (11). All PCR products were subcloned and sequenced as described previously (11).

RNA Dilution Experiments. Plasmids that contained PCR products subsequently identified as fragments corresponding to exons 3- and 4-deleted ER variant (D3-4-ER) and to variant deleted in exons 2, 3, and 7 (D2-3/7-ER) were linearized with *Bam*HI and gel purified as described previously (11). Corresponding sense RNAs were synthesized using Riboprobe Systems (Promega, Madison, WI) according to the manufacturer's instructions. One μg of total RNA from T-47D-5 cells was mixed with various amounts of synthetic D2-3/7-ER (ranging from 5 ng to 50 fg) or D3-4-ER RNA (50 fg). These spiked RNA samples were then reverse transcribed and amplified using 1/8U and 1/8L primers as described above.

Statistical Analysis. Each individual tumor sample was analyzed in at least three independent assays. Only bands reproducibly observed in three experiments were considered. The presence of a specific band in a tumor sample was scored only if its signal intensity placed it among the four strongest signals (as assessed by subjective visualization) observed in the corresponding lane. The tumor group in which the band corresponding to the WT-ER mRNA was detected (68 cases) presented the following characteristics: ER level ranging from 0 to 386 fmol/mg protein (average, 111 fmol/mg protein) and PR level ranging from 0 to 297 fmol/mg protein (average, 73 fmol/mg protein). For the purpose of analysis, this group was divided into two subgroups presenting ER, PR, or a grade above or below a point defined as the average of the ER value, PR value, or grade observed within the group. Possible associations between the detection of a particular variant and one particular subgroup were tested using either the χ^2 test, including Yates' correction when the estimated frequency was at least equal to 5, or the Fisher exact test (two tailed) in other cases.

Results

Coamplification of WT-ER mRNA and Deleted Variant mRNAs in Breast Tumor Samples. On the basis of the assumption that coamplification of WT-ER mRNA and variant ER mRNAs could effectively occur and therefore allow identification of the frequency and relative expression of variants in breast tumor tissues, 100 breast tumors were selected for analysis that represented a wide range of ER and PR levels, as measured by the ligand-binding assay, grade, nodal status, and size. Total RNA was extracted from each tumor sample and reverse transcribed. PCR was then performed using primers annealing with exon 1 and exon 8 sequences. Fig. 2 shows typical results obtained. Many different PCR products were observed in each of 70 ER-positive tumors but only in 3 of 30 ER-negative tumors. This difference did not result from variable input of cDNA, since similar signals were obtained in all samples after amplification of the housekeeping GAPDH cDNA (data not shown). Two bands that migrated with the apparent sizes of 1381 and 1197 bp were observed in most of the signal-positive tumors. These bands were detectable in 68 and 63 cases, respectively. Following subcloning and sequencing, these bands were shown to correspond to the WT-ER and an exon 7-deleted ER (D7-ER) variant mRNA, respectively. Six other bands that migrated at the apparent sizes of 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were consistently detected within the set of tumors studied, but at an apparently lower frequency. They were observed in 19, 8, 6, 11, 6, and 20 tumors and were found to correspond to ER variant mRNAs deleted in exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2–4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. Sequences of all of these variants, except D-3-7-ER variant, showed a perfect junction between exons surrounding the deletion area (data not shown). A 801-bp deletion was observed in the D-3-7-ER variant from nucleotides 931 to 1729 (22) located within exon 3 and exon 7, respectively. It should be stressed that some bands, either not consistently observed or specific for less than three tumors, have not yet been assessed further in this study.

Detection of a Particular Variant Depends on Its Initial Representation within the ER mRNAs Population. To determine whether the detection of a variant depended on its initial representation within the ER-like mRNA population, the balance of ER-deleted variants was artificially changed in favor of particular variants. Various amounts of synthetic RNAs corresponding to the D3-4-ER and D2-3/7-ER PCR products were added to total RNA extracted from T-47D-5 breast cancer cells. These RNA preparations were reverse transcribed and subsequently analyzed with PCR using 1/8U and 1/8L primers (Fig. 3). Bands corresponding to WT-ER, D7-ER, D4-ER, and D-3-7-ER were initially detected in T-47D-5. The addition of synthetic D2-3/7-ER RNA, which increased its ability to compete for the binding of 1/8U and 1/8L primers during the PCR reaction, drastically decreased signals corresponding to the initially detectable endogenous variants. The extinction of these signals was directly related to the concentration of the synthetic RNA added. The Addition

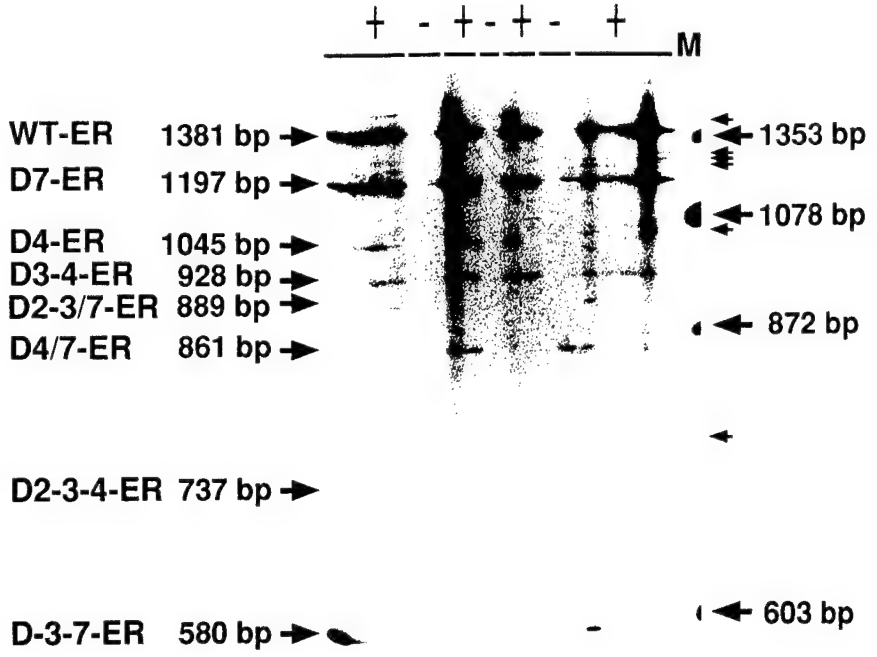

Fig. 2. Coamplification of WT-ER and deleted variant mRNAs in breast tumor samples: total RNA extracted from different ER-positive (+) and ER-negative (−) breast tumors was reverse transcribed and PCR amplified as described in "Materials and Methods" using 1/8U and 1/8L primers. Radioactive PCR products were separated on a 3.5% acrylamide gel and visualized using autoradiography. To each lane corresponds a unique tumor. Bands reproducibly obtained within the set of tumors studied and that migrated at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were identified as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2–4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. PCR products indicated by *small arrows*, barely detectable within the tumor population, *i.e.*, present in less than or equal to three particular tumors, have not yet been identified. *M*, molecular weight marker (ϕX174; Life Technologies, Inc., Grand Island, NY).

of two synthetic RNAs simultaneously resulted in the increased representation of two expected bands.

Detection of Particular Variants May Be Associated with Tumor Characteristics. Detection of ER variants using the approach described here appeared to depend on the initial relative ratio of expression between ER-like mRNAs. It was therefore of interest to search for possible associations between the detection of particular variants and other tumor characteristics. The detection of a specific band in a sample was defined here as its presence as one of the four main signals observed in the corresponding lane. The frequency of detection of each ER variant mRNA within tumors also expressing a detectable WT-ER band is presented Table 1. Using the mean ER, PR, and grade values as cutoff points for statistical analysis, we found that D-3-7-ER and D2-3-4-ER variants were preferentially detected in the subgroup with higher ER ($P < 0.001$) and higher grade ($P < 0.025$), respectively. D4-ER variant was more frequently observed in tumors of lower grade ($P < 0.05$) or with higher PR levels ($P < 0.01$).

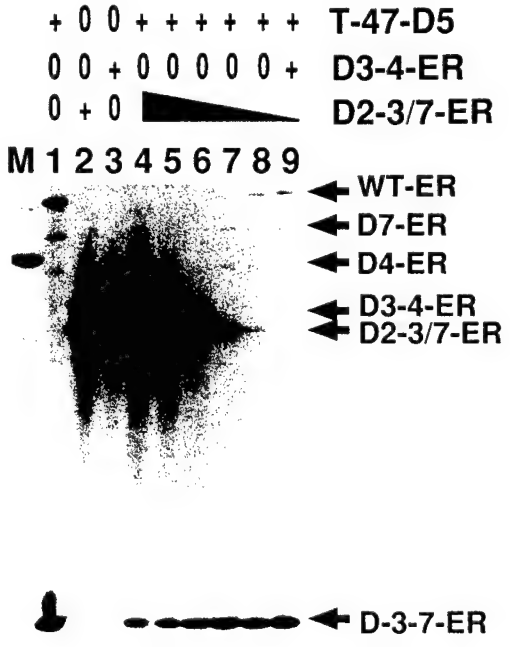

Fig. 3. Coamplification of WT-ER and deleted variant mRNAs after artificial modification of the balance between ER-like mRNAs: one μg of total RNA from T-47D-5 cells alone (*Lane 1*) or mixed with various amounts of synthetic D2-3/7-ER (5 ng, 500 pg, 50 pg, 5 pg, 500 fg, and 50 fg; *Lanes 4, 5, 6, 7, 8*, and *9*, respectively) and D3-4-ER (50 fg, *Lane 9*) RNAs. These spiked RNAs were then reverse transcribed and amplified as described in "Material and Methods." PCR products were separated on 3.5% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gel was dried and autoradiographed for 18 h. D2-3/7-ER and D3-4-ER RNAs alone (5 ng) were similarly analyzed (*Lanes 2* and *3*, respectively). *M*, molecular weight marker.

Discussion

We have used a new approach based on the competitive coamplification of WT-ER and exon-deleted or -inserted ER variant mRNAs to examine the overall expression of these two types of ER variants which encompass the majority of ER variant mRNAs thus far identified (23). Although another distinct group of variants, the truncated ER variants that include the widely expressed ER clone 4 variant (5, 18), cannot be assessed with this analysis, the strategy allows a broad investigation of the ER-like population and the integrity of the entire coding region within this species, without focusing on particular regions. This has enabled us to confirm the existence of four variants already described by others, *e.g.*, exon 7-deleted ER variant (3, 16), exon 4-deleted ER variant (7), exon 3–4-deleted ER variant (9), and a variant deleted in both exons 4 and 7 (17). Beyond these, three new variants were identified. Two of them, deleted in exons 2, 3, and 7 or exons 2–4, correspond to the usual exon-deleted ER variant pattern, *i.e.*, containing a perfect deletion of exon sequences. The third one contained part of exon 3 attached to a sequence beginning inside the seventh exon. It should be noted that very recently, Daffada and Dowsett (24) identified an ER variant presenting a similar pattern of intra-exon deletion between exons 4 and 7. Furthermore, we have been able to detect ER variant mRNA deleted in both exons 4 and 7 for the first time in multiple clinical material, supporting the potential relevance of such a variant *in vivo*. The function of the putative encoded protein which lacks a nuclear localization signal, all of the hinge domain, and is C-terminal truncated remains to be determined.

Using different RNA preparations, we showed that the detection of

Table 1 *Frequency of detection of ER variant mRNAs within 68 human breast tumors also expressing detectable WT-ER mRNA*

	Tumors expressing WT-ER	No. of tumors expressing detectable ER variant mRNAs						
		D7-ER	D4-ER	D3–4-ER	D2–3/7-ER	D4/7-ER	D2–3–4-ER	D–3–7-ER
ER < 111 (fmol/mg protein)	38	34	8	6	4	7	3	3
ER > 111 (fmol/mg protein)	30	27	9	2	2	3	2	16 $P < 0.001$[a]
PR < 73 (fmol/mg protein)	41	35	5	7	5	6	4	10
PR > 73 (fmol/mg protein)	27	26	12 $P < 0.01$[a]	1	1	4	1	9
4 ≤ grade ≥ 6	35	33	13 $P < 0.05$[a]	4	3	5	0	10
7 ≤ grade ≥ 9	33	28	4	4	3	5	5 $P < 0.025$[b]	9

WT and variant ER mRNAs were detected after co-amplification as described in "Materials and Methods."
[a] P values calculated using the χ^2 test with Yates' correction.
[b] P value calculated using the Fisher exact test (two tailed).

a variant depended on its initial representation within the ER-like mRNA population. The absence of a prominent signal corresponding to any particular variant could therefore result from its low relative representation. This could explain why variants deleted in either exon 3 or exon 5 were undetectable using our criteria and this approach, although their presence was confirmed by specific PCR amplification in some of the same tumors studied.[4] These variants may also correspond to infrequent or poorly represented ER-like mRNAs and therefore PCR products that we have not yet identified. On the other hand, the detection of any particular ER variant mRNA within a tumor sample can result from its overexpression or a change in the balance between all ER variant mRNAs. Using this approach, it is therefore possible to investigate the relative proportion of ER variant mRNAs, and also to compare breast samples regarding the relative expression of their ER-like mRNAs.

The set of tumors analyzed in this pilot study was chosen to obtain the widest qualitative representation of important breast tumor characteristics more than to establish statistical associations. The tumor population contained very different tumors spread over a wide range of ER and PR levels, size, grade, and nodal status. It was possible however to establish that detection of particular variants may be correlated with already known prognostic markers. It is interesting to note that the exon 4-deleted variant is associated in this study group with two different markers of good prognosis, *i.e.*, high PR and lower grade. This variant, initially described in breast cancer cell lines (7) and subsequently *in vivo* in several normal and tumor tissues (9, 10), is expected to encode an ER-like protein lacking most of the hinge domain, which includes an important nuclear localization signal and a part of the hormone binding domain. It might therefore have a cellular distribution and estrogen-binding affinity different from that of the WT-ER. Furthermore, the altered structure of this protein may lead to altered transcriptional activities.

The use of this approach to study a larger set of samples would allow the establishment of a typical pattern of ER variant mRNA expression for each type of tumor. Comparison of such patterns along with the subsequent analysis of the specifically detected transcripts could lead to the discovery of new prognostic factors and the identification of new contributors to tumor progression.

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[4] E. Leygue, unpublished data.

QUANTITATION OF A TRUNCATED ESTROGEN RECEPTOR VARIANT mRNA BY TRIPLE-PRIMER POLYMERASE CHAIN REACTION IN COMPARISON TO RNase PROTECTION ASSAY

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Running title: Triple-primer PCR in comparison to RNase protection assay.

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Category assignment:

Procedures of interest in the field of recombinant technonology and molecular genetics.

Abstract

Expression of clone 4 (C4) mRNA, a truncated variant form of the estrogen receptor (ER) mRNA increases during breast tumor progression. In order to compare human breast tissue samples regarding their C4 mRNA content relative to wild-type ER mRNA, we have recently developed an approach called Triple-Primer-Polymerase Chain Reaction (TP-PCR). This approach consists of co-amplification of wild-type ER (WT-ER) and C4 cDNAs using three primers in the PCR. The accuracy of this approach is here compared to that of a standardized RNase protection assay. Total RNA was extracted from twenty five human breast tumors with ER values ranging from 0 to 311 fmol/mg protein, as determined by ligand binding assay. A standardized RNAse protection assay allowed the determination, for eighteen tumors, of the absolute amount of WT-ER and C4 mRNAs in pg per mg of total RNA. A strong correlation ($r = 0.932$, $p < 0.0001$) is observed when comparing C4 mRNA expression relative to wild-type mRNA measured by RNase protection assay and TP-PCR. Moreover, when the sensitivity of the RNase protection assay did not allow the detection of C4 mRNA relative expression in seven tumors with ER values lower than 10 fmol/mg, TP-PCR allowed such an evaluation. TP-PCR appears therefore to be a good substitute for RNase protection assay to study the expression of truncated variant mRNAs in models where either the quantity of available material or the sensitivity of the RNase protection assay become limiting factors.

Introduction

Estrogen is a major regulator of growth and development of both normal and neoplastic human breast tissues. The action of estrogen is thought to be mainly mediated by the estrogen receptor (ER). This receptor, which belongs to the steroid/retinoic acid nuclear receptor superfamily, is divided into several structural and functional domains encoded by a mRNA containing 8 exons (1, 2). Several ER variant mRNAs have been identified in normal and neoplastic human breast tissue (3-8). These variant mRNAs, if translated, would encode ER-like proteins missing some regions of the wild-type (WT) molecule. Such proteins could therefore interfere with the wild-type ER signaling pathway, and alterations in ER variant expression may be involved in breast tumorigenesis (6, 9-14). Among the several described ER variant mRNAs, Clone 4 (C4) variant mRNA is the only one that has been isolated as a full length cDNA from a human breast cancer cDNA library (15). C4 mRNA consists of sequences from exon 1 and 2 of the WT-ER, followed by unrelated sequences. This truncated form of the WT-ER mRNA has been shown to be more highly expressed relative to the WT-ER transcript in tumors with parameters of poor prognosis and endocrine insensitivity (13). In order to quantify the expression of this transcript relative to WT-ER mRNA using small amounts of breast tissue specimens, we have recently developed (16) an approach called triple-primer polymerase chain reaction (TP-PCR). In this approach, depicted in Figure 1, three primers are used in the PCR. The upstream primer (ERU) recognizes both the C4 and the wild-type cDNAs. The two downstream primers (ERL, C4L) are specific for the WT cDNA and C4 cDNA, respectively. Since the upstream primer anneals to both cDNAs, TP-PCR leads to a competitive amplification of truncated and wild-type cDNA, the final ratio between the co-amplified products being related to the initial input cDNA ratio.

The accuracy of this approach to evaluate the ratio between C4 and WT-ER transcripts within a panel of human breast tumors with various ER values, as determined by ligand binding assay is here compared to that of a standardized RNAse protection assay.

Materials and methods

Human breast tissues

Human breast tumor specimens (25 cases) were obtained from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Twenty tumors were ER positive, as determined by ligand binding assay, with values ranging from 4.5 to 311 fmol/mg protein (median= 93 fmol/mg). The five remaining cases were ER negative, as determined by ligand binding assay, with values ranging from 0 to 1.8 fmol/mg protein (median 0.9 fmol/mg).

RNA Extraction and RNase protection assay

Total RNA was extracted from frozen tissues using guanidium-thiocyanate as previously described (4). Antisense riboprobes spanning the point at which the clone 4 truncated ER mRNA sequence diverges from the WT-ER mRNA sequence (15) were synthesized as previously described (17). The level of clone 4 truncated ER mRNA and WT-ER mRNA in 10 µg total RNA was determined using an RNase Protection Assay kit (RPA II, Ambion) following the manufacturers instructions. Briefly, RNA was denatured at 80°C for 5 min in the presence of 5 x 10^5 dpm of ^{32}P-labelled riboprobe, then hybridized at 42°C for 16 hours. Following RNase digestion, samples were electrophoresed on 6% acrylamide gels containing 7 M urea, dried and autoradiographed.

Reverse transcription and TP-PCR

For each sample, one microgram of total RNA was reverse transcribed in a final volume of 15 µl as described previously (8). One microliter of the reaction mixture was taken for subsequent amplification. Three primers were used in this study (Figure 1). ERU (5'-TGTGCAATGACTATGCTTCA-3', sense, located in WT-ER exon 2; 792-811) and ERL (5'-GCTCTTCCTCCTGTTTTTAT-3', antisense, located in WT-ER exon 3; 921-940) allowed amplification of a 148 bp fragment corresponding to WT-ER mRNA. C4 specific primer (C4L, 5'-TTTCAGTCTTCAGATACCCCAG-3', antisense; 1315-1336) was chosen as spanning the only region of the C4 unique sequence that does not present any homology with repetitive LINE-1 sequences (15). ERU and C4L allowed amplification of a 536 bp fragment corresponding

specifically to clone 4 truncated ER variant mRNA. Positions given correspond to published sequences of ER cDNA (18) for ERU and ERL and of clone 4 cDNA (15) for C4L primer.

PCR amplifications were performed in a final volume of 10 µl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer (ERU, ERL and C4L), 1 unit of Taq DNA polymerase (GIBCO-BRL) and 10 nM of dCTP [α-^{32}P] (ICN Pharmaceuticals Inc, Irvine, California). Each PCR consisted of 30 cycles (1 minute at 94°C, 30 seconds at 60°C and 1 minute at 72°C) using a Thermocycler (Perkin Elmer). 4 µl of the reaction was then denaturated by addition of 6 µl of 80% formamide buffer and boiling before electrophoresis on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with two intensifying screens for 2 hours.

Quantification

RNase protection assay

To quantify C4 and WT-ER mRNAs within breast tumor samples, a standard curve was established in each assay. Clone 4 and WT-ER mRNAs (30, 125, 500 pg clone 4 RNA and 125, 500, 2000 pg WT-ER RNA) synthesized using T7 RNA Polymerase were purified on a Sephadex G-50 column and quantitated spectrophotometrically. WT-ER RNA was transcribed from linearized pOR-8, which contains the entire WT-ER coding sequence but is missing the 3'-untranslated portion of the ER mRNA (kindly provided by P. Chambon, 18). Full-length C4 RNA was transcribed from linearized pSK-C4 (15). Standard RNAs were analyzed together in the same assay as the breast tumor mRNAs. Bands corresponding to the clone 4 variant ER mRNA and WT-ER mRNA protected fragments were excised from the gel and counted after addition of 5 ml scintillant (ICN Pharmaceuticals, Inc., Irvine, CA) in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). For each sample, absolute amounts of clone 4 and WT-ER mRNA were determined from the standard curve.

TP-PCR

The approach used to evaluate the C4 variant mRNA expression relative to WT-ER mRNA has already been described (16). PCR co-amplification of WT-ER and C4 variant generates 2 bands (Figure 1) whose ratio is constant with varying cycle number and is independent of initial input cDNA (16). Bands corresponding to C4 and WT mRNAs were excised from the gel and corresponding signals were subsequently measured by scintillation counting. The C4 signal was expressed as a percentage of the WT-ER signal. For each sample, at least 2 independent TP-PCR assays were performed.

Results and Discussion

Total RNA was extracted from frozen tissues from twenty five human breast tumors with ER values ranging from 0 to 311 fmol/mg, as determined by ligand binding assay. Ten micrograms of each RNA sample were analyzed in a standardized RNase protection assay in order to determine the absolute amount of C4 and WT-ER mRNAs within each sample. A typical autoradiogram of such an assay is shown in Figure 2. The signals corresponding to C4 and WT-ER mRNAs were quantified as described in the Materials and Methods section. In each assay, known amounts of synthetic WT-ER and C4 mRNAs were analyzed in parallel in order to establish a standard curve (Figure 3) allowing the determination of absolute levels of C4 and WT-ER mRNAs, expressed as pg/10µg RNA (Table 1). Because of the very low C4 protected fragment signal ($\leq$ 15 dpm) in seven tumors, it was not possible to determine confidently the absolute amount of C4 mRNA in these samples (not determined, nd). All C4 negative tumors by RNase protection assay were from tumors with ER values lower than 10 fmol/mg, as determined by ligand binding assay. For the eighteen remaining samples, ER values as determined by ligand binding assay ranged from 1.2 to 311 fmol/mg. The absolute amounts of C4 and WT-type ER mRNAs were determined by RNase protection assay and varied from 2 to 83.9 pg/10µg RNA and from 9 to 3651 pg/10µg RNA, respectively. For each sample, the C4 mRNA signal was expressed as a percentage of the WT-ER mRNA signal (Table 1).

The lack of sensitivity of the RNase protection assay for a subset of tumors with very low (<10 fmol/mg) ER values by ligand-binding assay is an important limiting factor. It effectively means that in a screening study, ER negative tumors (<3 fmol/mg protein) as well as ER positive tumors with ER values lower than 10 fmol/mg, as measured by ligand binding assay, cannot be reliably assessed for C4 mRNA expression by RNase protection assay . This together with the relatively large amount of RNA needed to perform an RNase protection analysis severely limits the usefulness of a standardized RNase protection assay in such screening studies.

In order to determine if an already described TP-PCR technique might provide a more practical alternative to the RNase protection assay, C4 mRNA relative expression was determined by TP-PCR within the same twenty five RNA samples. One microgram of total RNA extracted from each breast tumor sample was reverse-transcribed and an aliquot of the cDNA amplified by TP-PCR in the presence of dCTP [α-^{32}P] as described in the Materials and Methods section. PCR products were separated on 6% acrylamide denaturating gels. A typical autoradiogram is shown Figure 4. Both C4 and WT-ER cDNAs signals were detected in all twenty five tumors studied, independent of their ER status as determined by ligand binding assay. It should be stressed that an additional band can be observed in most of the samples. This band has been identified after subcloning and sequencing to be a product of an exon 2 duplicated ER variant mRNA. The intensity of the signal obtained from this exon 2 duplicated ER band parallels that of the WT-ER band. C4 and WT-ER signals were quantified as described in the Materials and Methods section. The signal corresponding to C4 was expressed as a percentage of the WT-ER signal. Table 1 presents the average of a least two independent TP-PCR experiments.

Linear regression analysis (Figure 5) shows a highly significant correlation between C4 mRNA relative expression as determined by RNase protection assay (in the eighteen tumors where a C4 signal was detectable) and C4 mRNA relative expression determined by TP-PCR ($r = 0.932$, $P < 0.0001$). This correlation between the RNase protection assay and TP-PCR results suggests that TP-PCR evaluation of the C4 mRNA relative to WT-ER mRNA expression is accurate and can be used to compare breast tissue

samples. The co-amplification of the exon 2 duplicated ER variant mRNA using TP-PCR does not seem to interfere with the relationship between TP-PCR and RNase protection assay. One should note that because of the design of the riboprobe used (covering WT-ER exon 2 sequence and C4 specific sequence), the protected fragment from this exon 2 duplicated RNA would be included in the WT-ER signal using the RNase protection assay. In addition because the upstream primer in the TP-PCR is situated in exon 2, amplification of the exon 2 duplicated ER results in both WT-ER and exon 2 duplicated sized products. Similarly, because several exon deleted ER variants have been observed in human breast tissues (5-14), one should note that whether RNase protection assay or TP-PCR is used, the signal attributed to WT-ER mRNA corresponds to a signal representing to all ER-like mRNA containing the exon 2 and exon 3 sequences. The values obtained using both techniques are therefore representative of the balance existing between C4 mRNA and ER-like mRNAs, including WT-ER but also exon7-deleted, exon 5-deleted, exon 4-7-deleted etc... mRNAs. Interestingly, correlation between relative measurements of the mRNA levels of WT-ER and C4 using RNase protection assay and TP-PCR are in better agreement with each other than is the measurement of WT-ER by RNase protection assay compared with the ligand-binding assay (Table 1). This may in part be due to tumor heterogeneity, ie., the portion of the tumor used in the ligand-binding assay may have had a much different cellular composition (normal and neoplastic epithelial cells, normal stroma, myoepithelial cells) than the portion used for RNA extraction. Another possible explanation is that the ligand-binding assay is in fact a measurement of an ER protein which contains a functional ligand-binding domain, and some tumors might be expressing higher levels of variant ERs which are lacking a hormone-binding domain. This hypothesis is supported by a recent study in which a subset of human breast tumors was identified which presented stronger signals using an antibody recognizing an N-terminal epitope of the ER than with an antibody recognizing a C-terminal epitope (19). These tumors were shown to express relatively higher levels of variant ER mRNAs, including C4 variant ER mRNA, which if translated would be missing the ligand-binding domain of the ER and would not be detected in a ligand-binding assay.

To our knowledge this study is the first that addresses the question of the comparison of an already established quantitative approach such as the RNase protection assay with an RT-PCR based approach in the study of ER variant mRNA expression. All of the studies published so far have either been done by RNase protection assay alone or by RT-PCR alone. Considering the clinical relevance of the measurement of the relative level of these variants with respect to WT-ER within human breast tissue samples and the sensitivity of an RT-PCR based approach, such a comparative study was deemed necessary.

The low amount of starting material needed, together with the higher sensitivity observed (samples C4 negative by RNase protection assay presented detectable levels of C4 and WT-ER mRNA by TP-PCR) make TP-PCR an attractive alternative to the RNase protection assay in studies where such factors are limiting. It should be underlined that this TP-PCR approach can be adapted to all models where the relative quantification of two RNAs sharing one common sequence (initiated from different promoters, for example) is of importance.

Acknowledgements

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Legends to Figures

Figure 1: Schematic representation of Triple Primer-Polymerase Chain Reaction principle (TP-PCR) and RNase protection assay

Three primers are used simultaneously during the PCR. The upper primer (ERU) recognizes both wild-type ER cDNA and truncated C4 cDNA. The lower primers (ERL and C4L) are specific for each cDNA. Competitive amplification of a 148 bp and a 536 bp fragments occurs, corresponding to WT-ER and C4 cDNA, respectively. The final PCR-product ratio is related to the initial input cDNAs ratio. This ratio is constant with varying cycle number and independent of initial input cDNA (16).

RNase protection assay is performed using a probe covering a part of the exon 2 of WT-ER molecule and sequences C4 specific. The protected fragments are 257 bp and 302 bp long for WT-ER and C4 respectivly.

Figure 2: Quantification of C4 and WT-ER mRNAs by RNase protection assay

Ten micrograms of RNA extracted from twenty five breast tumor samples were analyzed as described in the Materials and Methods section. In each experiment, a synthetic RNA preparation (S1: 30 pg C4 mRNA, 125 pg WT-ER mRNA; S2: 125 pg C4 mRNA, 500 pg WT-ER mRNA; S3: 500 pg C4 mRNA, 2000 pg WT-ER mRNA) was analyzed in parallel to establish a standard curve. The bands migrating at 257 bases and 302 bases correspond to WT-ER and C4 mRNAs, respectively. Signals were counted and absolute amounts of C4 and WT-ER mRNA determined as indicated in the Materials and Methods section. The film presented was overexposed to allow the visualization of the clone 4 protected fragment in most of the samples.

Figure 3: Standard curve allowing the subsequent quantification of C4 and WT-ER mRNA and their relative expression within breast tumor samples by RNase protection assay

Signals corresponding to C4 and WT-ER mRNAs standard dilutions (S1, S2 and S3, Figure 2) were measured and a standard curve established. Signals measured for each sample were compared to this curve

generated for each assay, in order to quantify C4 and WT-ER mRNAs in each tumor. Results are expressed as pg/10 μg of total RNA.

Figure 4: Determination of C4 mRNA relative expression by TP-PCR

For each tumor, 1 μg of RNA was reverse-transcribed before performing TP-PCR in the presence of dCTP [α-^{32}P] as described in the Materials and Methods section. PCR products were separated by PAGE and corresponding signals quantified as described in the Materials and Methods. The bands migrating at 148 bp and 536 bp correspond to WT-ER and C4 cDNAs, respectively. M: molecular size marker (Φx174, Gibco BRL, Grand Island, NY).

Figure 5: Linear regression analysis of C4 expression (expressed as a percentage of the corresponding WT-ER expression) as determined by TP-PCR versus standardized RNase protection assay in eighteen human breast tumors

Table legends

Table 1: C4 and WT-ER mRNA expression in twenty five human breast tumors, as determined by RNase protection assay and TP-PCR

[a] not determined

Sample N°	Ligand Binding	RNase protection		TPPCR	
	ER fmole mg	C4 pg/10µg	WT-ER pg/10µg	C4 %	C4 %
5	0.0	nd[a]	nd	-	1.7
3	0.4	nd	nd	-	2.6
1	0.9	nd	nd	-	3.1
24	1.2	6.2	105.1	5.9	3.3
4	1.8	nd	nd	-	3.7
23	4.5	10.0	54.3	18.4	22.7
8	5.8	nd	26.8	-	2.8
7	6.3	nd	224.6	-	3.4
2	8.7	nd	9.0	-	2.2
19	10.0	22.6	902.9	2.5	3.6
10	17.8	5.3	146.4	3.6	4.1
13	25.0	2.3	112.0	2.0	1.0
15	44.0	5.0	148.5	3.4	5.9
22	57.0	11.8	153.6	7.7	14.1
11	90.0	2.5	129.1	1.9	1.7
21	96.0	9.6	263.4	3.6	2.2
14	105.0	4.6	94.4	4.9	5.0
17	111.0	26.7	320.3	8.3	9.1
9	121.0	4.6	277.7	1.7	2.4
6	146.0	2.0	105.0	1.9	1.9
18	198.0	15.8	422.0	3.7	7.0
20	236.0	8.8	288.4	3.0	3.5
12	289.0	3.6	80.5	4.5	8.0
16	304.0	38.8	1440.8	2.7	3.7
25	311.0	83.9	3651.0	2.3	3.2

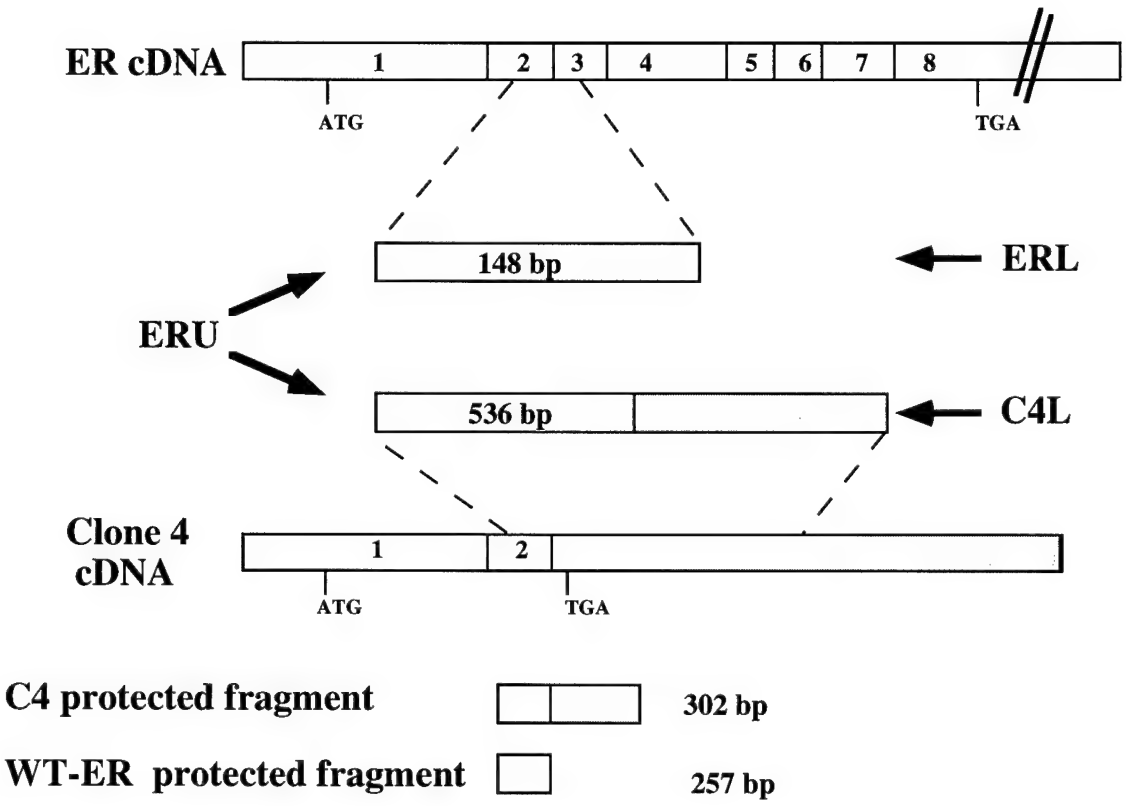

ER cDNA
1
2
3
4
5
6
7
8
ATG
TGA
148 bp
ERL
ERU
536 bp
C4L
Clone 4
cDNA
1
2
ATG
TGA
C4 protected fragment
302 bp
WT-ER protected fragment
257 bp

Figure 1

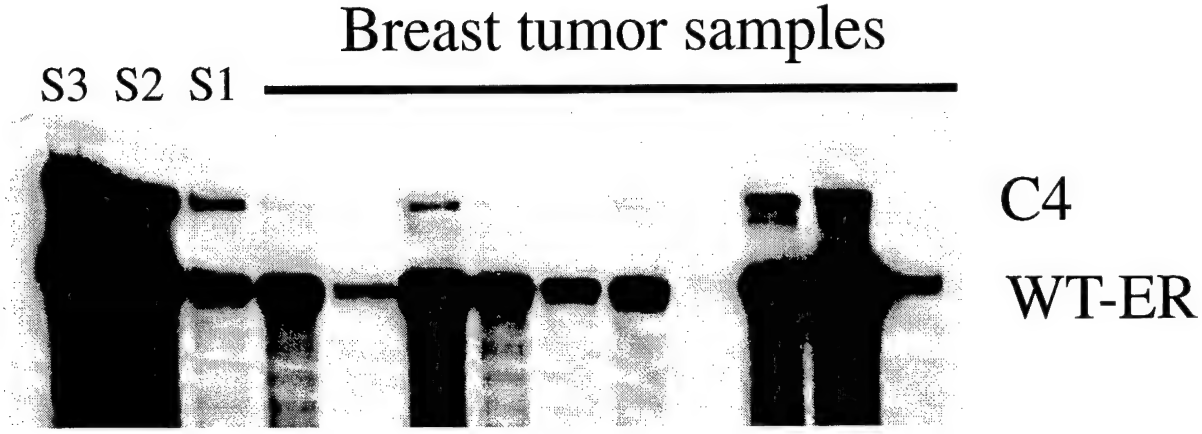


Figure 2

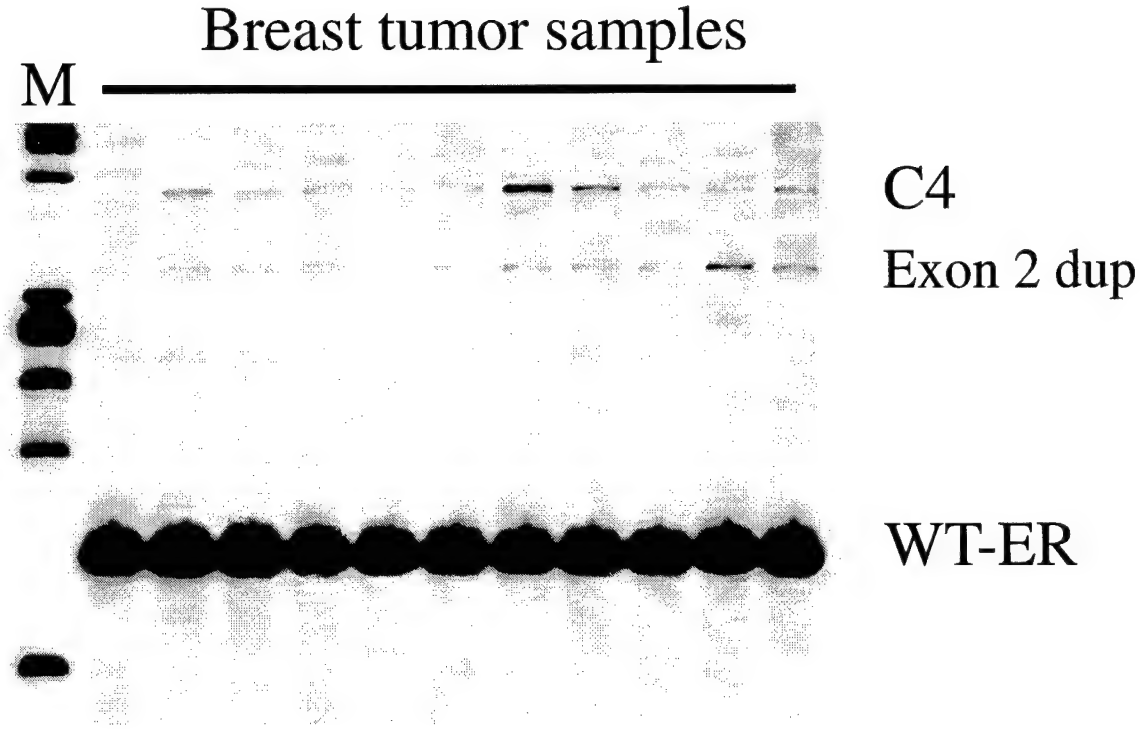
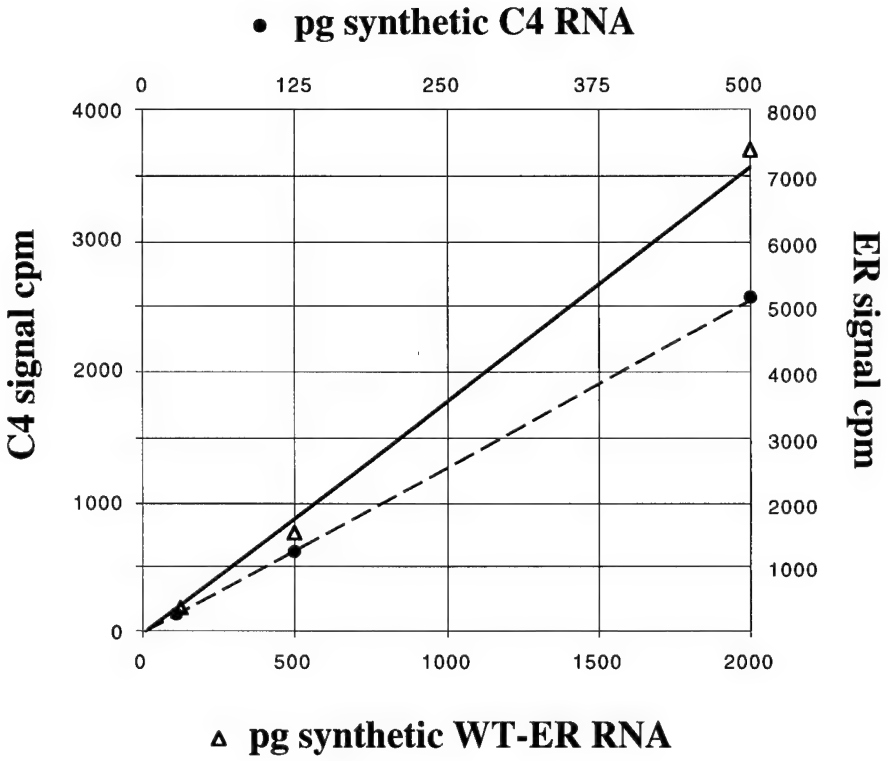


Figure 4


• pg synthetic C4 RNA
0
125
250
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1500
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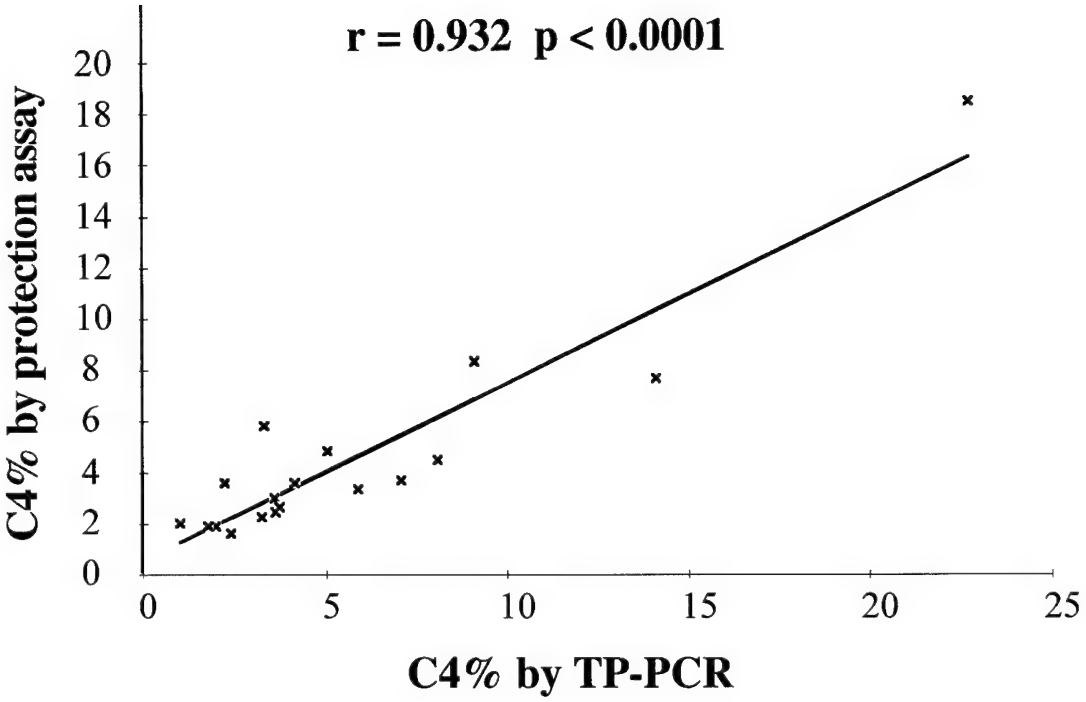
C4 signal cpm
ER signal cpm
△ pg synthetic WT-ER RNA

Figure 3


r = 0.932 p < 0.0001
C4% by protection assay
C4% by TP-PCR
0
2
4
6
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5
10
15
20
25

Figure 5

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Expression of Estrogen Receptor Variant Messenger RNAs and Determination of Estrogen Receptor Status in Human Breast Cancer

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Estrogen receptor (ER) status of breast cancer can be assessed by immunohistochemical assay (IHA), although we have previously observed that ER-IHA levels can be inconsistent between amino-terminal and carboxyl-terminal-targeted antibodies. To address the hypothesis that this discrepancy is attributable to expression of ER variant mRNAs encoding truncated ER-like proteins, we have studied 39 IHA-consistent and 24 IHA-inconsistent breast tumors by reverse transcription polymerase chain reaction to examine the expression of multiple exon-deleted (D-ER) variant mRNAs and the truncated ER clone 4 variant mRNA. ER variants D7-ER, D4-ER, D3–4-ER, and D4–7-ER were detected at similar frequencies in both groups. However, ER variants D2–3/7-ER, D2–3-4-ER ($P < 0.05$), and D-3–7-ER ($P < 0.01$), which encode putative short ER-like proteins that might be recognized only by an amino-terminal-targeted antibody, were preferentially detected in inconsistent cases. ER clone 4 mRNA expression was also higher in inconsistent tumors ($P < 0.001$). Further analysis showed that, whereas overall prevalence of ER variant mRNAs was similar in both tumor groups, occurrence of the subset of variant mRNAs encoding putative truncated proteins was also higher in IHA-inconsistent tumors ($P < 0.05$). These data suggest that ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER-IHA levels determined using amino- or carboxyl-terminal-targeted antibodies. (Am J Pathol 1997, 150:1827–1833)

Estrogen receptor (ER) determination is an important parameter in the clinical management of breast cancer.[1,2] Until recently, ER content was assessed principally by ligand-binding techniques such as dextran-coated charcoal (DCC) or sucrose gradient assays. Now, with the development of several antibodies able to recognize ER protein, immunohistochemical assay (IHA) has become an alternative approach to determine ER status of breast tumors and to predict endocrine response in breast cancer.[3,4] The ER-IHA approach has significant advantages including the potential for parallel assessment of tumor cell content and heterogeneity of ER expression. However it differs from traditional methods in that ER activity is defined by structural rather than functional criteria.

ER-IHA in tissue sections has been successfully achieved by several different antibodies, including 1D5, H222, and AER311, which are able to recognize different epitopes within particular domains of the ER protein (Figure 1).[3–5] However, we and others have previously observed that the ER-IHA results from some tumors are discordant between different antibodies that are able to recognize either the NH_2 or the COOH terminals, with a tendency to higher signals with NH_2-terminal-targeting antibodies.[4,5] Although these differences might relate to different antibody affinities, another explanation lies in the existence of ER variants. Beside the wild-type ER mRNA transcript, several ER variant mRNAs have been described in both normal and cancer tis-

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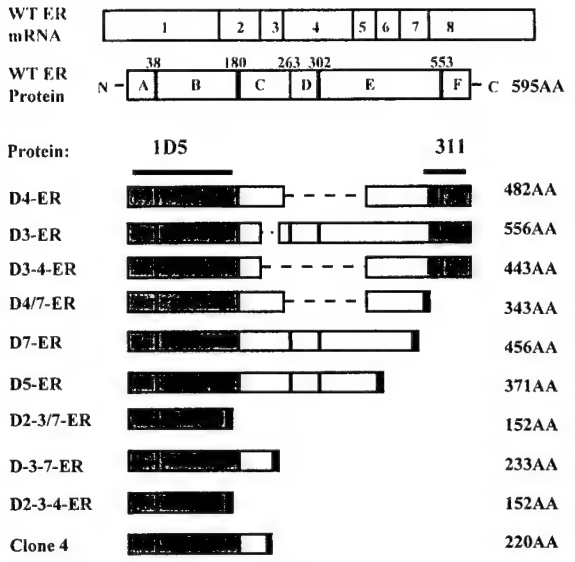

Figure 1. *Schematic presentation of WT-ER protein and the predicted proteins encoded by ER variant mRNAs. ER protein contains A to F functional domains. Region A/B of the receptor is implicated in transactivating function (TAF1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (TAF2). WT-ER reading frame is conserved in ER variant mRNAs deleted in exon 4, in exon 3, and in both exons 3 and 4. Encoded proteins from D4-ER, D3-ER, and D3–4-ER, respectively, are similar to WT-ER* (open box) *but miss some internal amino acids* (- - -). *Simple deletion of exon 7 or exon 5 and multiple deletion of exon 4 and exon 7; exons 2, 3, and 7; exons 3 and 7; and exons 2, 3, and 4 introduce a shift in the ER-WT reading frame. The resulting proteins, D7-ER, D5-ER, D4/7-ER, D2–3/7-ER, D-3–7-ER, and D2–3-4-ER, respectively, are therefore similar to WT-ER* (open box) *but are truncated of the C-terminal WT region* (black box, *indicating amino acids different from WT-ER). Clone 4 protein is encoded by an ER variant mRNA containing WT-ER exon 1 and exon 2 juxtaposed with line-1-related sequences. Clone 4 protein is similar to WT-ER* (open box) *but is missing the C terminal. The gray areas represent regions of the protein that are theoretically recognized by 1D5 or AER311 antibodies.*

sues.[6–14] Most of these variants are suspected to result from alternative splicing of WT-ER mRNA and consist of exon-deleted and truncated variants.[6,8] Figure 1 shows some of the putative proteins encoded by these variants and illustrates that, whereas some of these altered proteins may still possess both NH_2- and COOH-terminal epitopes of the wild-type (WT) protein, others will be truncated and lack the COOH terminal as a result of an exon deletion that introduces a shift in the reading frame. In addition to exon-deleted ER mRNA variants, several truncated variants have been described, among which the ER clone 4 variant is highly prevalent in breast tumors.[8] The sequence of this variant mRNA corresponds to WT-ER exon 1 and 2 juxtaposed to line-1-related sequences, and *in vitro* analysis shows that it encodes a putative ER-like protein missing the carboxyl-terminal extremity.

To address the hypothesis that discrepancies observed by IHA using 1D5 and AER311 antibodies in breast tumors could result from particular ER variant expression, we investigated 39 IHA-consistent and 24 IHA-inconsistent breast tumors for the most prevalent exon-deleted ER variant mRNAs and in parallel for the level of ER clone 4 truncated variant mRNA expression by two reverse transcriptase polymerase chain reaction assays that we have recently developed to assess multiple ER variants in breast cancer tissues.[15,16]

Materials and Methods

Human Breast Tissues and ER Status Determination

The study was carried out on 63 cases of invasive ductal and invasive lobular breast carcinomas obtained from the NCIC-Manitoba Breast Tumor Bank.[17] These cases correspond to the ER-positive subset of a series of 97 tumors previously studied by IHA.[5] In all cases, the specimens had been rapidly frozen at −70°C as soon as possible after surgical removal. Subsequently, a portion of the frozen tissue from each case was processed routinely to create formalin-fixed, paraffin-embedded tissue blocks that were matched and orientated relative to a corresponding frozen tissue block. Paraffin sections were previously analyzed by IHA using 1D5 (Dako, Dimension Labs, Mississauga, Canada) and AER311 (Neomarkers, Lab. Vision Corp., Fremont, CA) ER monoclonal antibodies.[5] In each case, immunohistochemical staining was assessed, without knowledge of the ER DCC status or antibody used, by a semiquantitative H score system (range, 0 to 300) for both antibodies and in the same regions on adjacent serial sections. When a difference of H score values between the two antibodies was >50, tumors were classified as inconsistent. When the difference of H score values was <50, the tumors were considered as consistent. Overall, the mean ER and progesterone receptor (PR) status and the distribution of ER and PR levels between the inconsistent and consistent groups was very similar (see Table 1). Within the inconsistent tumor group (24 cases), 8 tumors were low ER positive (3 to 10 fmol/mg protein; 33%), 6 tumors were middle ER positive (11 to 50 fmol/mg protein; 25%), and 10 were high ER positive (>50 fmol/mg protein; 42%), as determined by ligand-binding assay. Within the consistent tumor group (39 cases), 6 cases were low ER positive (15%), 12 cases were middle ER positive (31%), and 21 were high ER positive (54%).

Table 1. *Number of Tumors Expressing Detectable ER Variant in Consistent and Inconsistent Tumors*

Tumors	n	ER^{DCC}	PR^{DCC}	D7-ER	D4-ER	D3-4-ER	D4/7-ER	D2-3/7-ER	D2-3-4-ER	D-3-7-ER	ER $V.^{OF}$	ER $V.^{IF}$
Consistent	39	81 (89)	60 (75)	35	9	3	4	0	0	2	6	12
Inconsistent	24	69 (86)	55 (69)	22	6	2	2	2	3	8	10	8
P				>0.05	>0.05	>0.05	>0.05	>0.05	<0.05	<0.01	<0.05	>0.05

Analysis was by χ^2. ER $V.^{OF}$, out-of-frame exon-deleted ER variant mRNAs excluding D7-ER; ER $V.^{IF}$, in-frame exon-deleted ER variant mRNAs; ER^{DCC}, mean (SD) ER status measured by DCC assay (fmol/mg protein); PR^{DCC}, mean (SD) PR status measured by DCC assay (fmol/mg protein).

Extraction of mRNA and Reverse Transcription

For each case, a specific face of a frozen tissue block that matched the corresponding face of the paraffin block previously studied by IHA was selected.[17] Total RNA was extracted from histologically defined regions within 20-μm cryostat sections of frozen tissue using a small-scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH) as previously described.[18] Reverse transcription reactions were performed in triplicate in a final volume of 15 μl,[13,15] and 1 μl of the reaction mixture was taken for subsequent PCR amplification in either long-range PCR or triple-primer PCR assays described below.

Analysis of Prevalence of ER Variant mRNAs

Prevalence of ER variant mRNAs within breast tumor samples was assessed by PCR analysis performed by a long-range PCR assay as previously described.[15] The primers used consisted of 1/8U primer (5′-TGCCCTACTACCTGGAGAACG-3′, sense, located in WT-ER exon 1) and 1/8L primer (5′-GCCTCCCCCGTGATGTAA-3′, antisense, located in WT-ER exon 8). This primer set allowed amplification of a 1381-bp fragment corresponding to WT-ER mRNA and all deleted or inserted ER variant mRNAs containing exon 1 and exon 8 sequences. PCR amplifications were performed in a final volume of 10 μl, in the presence of 10 nmol/L [α-^{32}P]dCTP (ICN Pharmaceuticals, Irvine, CA), 4 ng/μl each primer, and 1 U of *Taq* DNA polymerase (Promega, Madison, WI). Each PCR consisted of 40 cycles (1 minute at 60°C, 2 minutes at 72°C, and 1 minute at 94°C) using a thermal cycler (MJ Research PT100, Fisher Scientific, Ottawa, Canada). After PCR, 2 μl of the reaction was denaturated in 80% formamide buffer, and the PCR products were separated on 3.5% polyacrylamide gels containing 7 mol/L urea (PAGE). After electrophoresis, the gels were dried and autoradiographed for 18 hours. Identities of specific bands were then confirmed by reference to size markers, subcloning, and sequencing.[13]

Quantification of ER Clone 4 mRNA Expression

Quantification of clone 4 mRNA expression was performed using a triple-primer PCR assay as previously reported.[16] Briefly, three primers, E2U (5′-AGGGTGGCAGAGAAAGAT-3′, sense, located in WT-ER exon 2), E3L (5′-TCATCATTCCCACTTCGT-3′, antisense, located in WT-ER exon 3), and C4L (5′-GGCTCTGTTCTGTTCCATT-3′, antisense), were used during PCR, performed in the presence of [α-^{32}P]dCTP. These primers allowed the co-amplification of a 281-bp and a 249-bp fragment corresponding to WT-ER and clone 4 truncated ER variant mRNAs, respectively. PCR products were separated by PAGE. After electrophoresis, gels were dried and autoradiographed. Autoradiographs were analyzed with a video-densitometry system and quantitated using MCID M4 software (Imaging Research, St. Catherines, Canada). The signal corresponding to ER clone 4 was measured relative to expression of the corresponding WT-ER and expressed as a percentage relative to a reference standard (an ER-positive tumor sample) to reduce any variation due to signal intensity in different gels. ER clone 4 expression was determined from the mean of three independent RT-PCR assays performed without knowledge of the IHA status. Means obtained from the 24 IHA-inconsistent tumor samples were then compared with those found in the 39 IHA-consistent tumor samples using the Mann-Whitney rank sum test (two sided).

Results

Detection of Exon-Deleted ER Variant mRNAs within Consistent and Inconsistent Tumors

Prevalence of exon-deleted ER variant mRNAs was investigated within 63 breast tumors, previously studied by IHA using 1D5 and AER311 antibodies[5] and subsequently classified as IHA consistent (39 cases) or IHA inconsistent (24 cases) as illustrated in

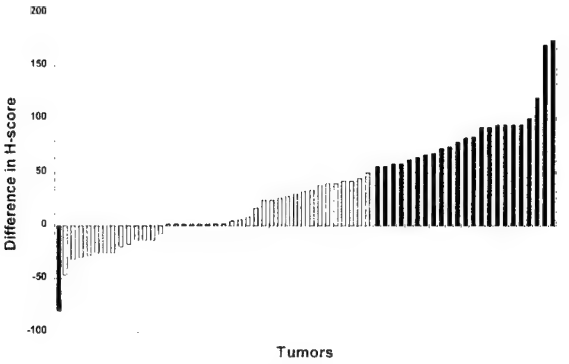

Figure 2. *Graph to show the difference in H score (IHA-1D5 to IHA-311) for each of 63 tumors showing the basis for classification into IHA consistent (< 50 H score difference;* white bars*) and IHA inconsistent tumors (> 50 H score difference;* black bars*).*

Figure 2. Long-range RT-PCR assay using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences first allowed assessment of the most prevalent exon-deleted variant mRNAs in comparison with the co-amplified WT-ER mRNA, as described previously.[15] Several different PCR products were observed within the set of tumors studied (Figure 3) that have previously been shown to correspond to the WT-ER (1381 bp) and ER variant mRNAs deleted in exon 7 (D7-ER, 1197 bp), exon 4 (D4-ER, 1045 bp), both exons 3 and 4 (D3–4-ER, 928 bp), exons 2, 3, and 7 (D2–3/7-ER, 889 bp), both exons 4 and 7 (D4/7-ER, 861 bp), exons 2, 3, and 4 (D2–3-4-ER, 737 bp), and within exon 3 to within exon 7 (D-3–7-ER, 580 bp), respectively.[15] Results obtained for IHA-consistent and IHA-inconsistent tumor subgroups are summarized in Table 1. D7-ER, D4-ER, D3–4-ER, and D4/7-ER variant mRNAs were detected at the same frequency in both subgroups. However, D2–3/7-ER, D2–3-4-ER, and D-3–7-ER mRNAs were preferentially detected in IHA-inconsistent tumors. This increased prevalence reached statistical significance for both D2–3-4-ER and D-3–7-ER mRNAs ($P < 0.05$ and $P < 0.01$). Given that the D7-ER variant was detected uniformly (>90%) in both subgroups, we chose to assess the remainder of the variant mRNAs that were not uniformly detected (ie, all variants except D7-ER). These were then considered with respect to the putative ER-like protein they should encode and classified further into two subgroups. In-frame variants (ER V.^{IF}) comprised those with a sequence modification that did not introduce a shift in the reading frame and that could encode proteins theoretically recognized by both 1D5 and AER-311 antibodies (D4-ER and D3–4-ER variant mRNAs). Out-of-frame variants (ER V.^{OF}) comprised variants encoding proteins theoretically only recognized by 1D5 antibody (D4/7-ER, D2–3/7-ER, D2–3-4-ER, and D-3–7-ER). ER V.^{IF} were detected in 12 (31%) and 8 (33%) IHA-consistent and IHA-inconsistent tumors, respectively. At the same time, ER V.^{OF} were detected in only 6 (15%) IHA-consistent compared with 10 (42%) IHA-inconsistent tumors, respectively ($P < 0.05$, χ^2 analysis).

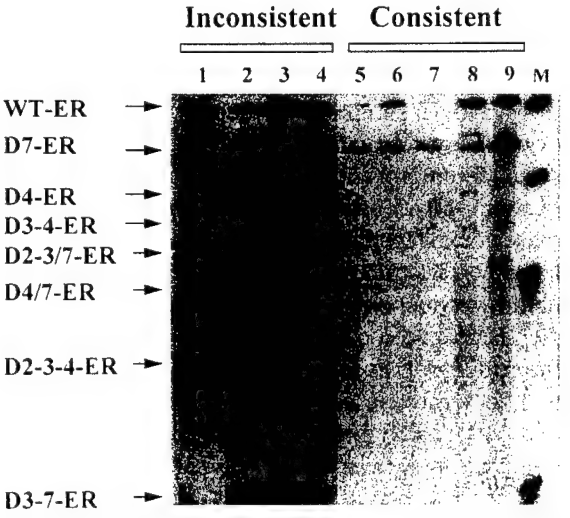

Figure 3. *Comparison of exon-deleted ER variant expression between IHA-consistent[1–4] and IHA-inconsistent[5–9] breast tumors. Total RNA was extracted from inconsistent and consistent tumors, reverse transcribed, and subsequently amplified by PCR as described in Materials and Methods. PCR products were separated on PAGE and visualized by autoradiography. Bands migrating at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were identified by isolation and sequencing as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3–4-ER), exons 2, 3, and 7 (D2–3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3, and 4 (D2–3-4-ER), and within exon 3 to within exon 7 (D-3–7-ER), respectively. M, molecular weight marker (ϕ X174, Gibco BRL, Grand Island, NY).*

Quantification of Clone 4 mRNA Expression

Expression of a prevalent truncated ER mRNA variant, the ER-clone 4 variant, which is also suspected to encode a truncated ER-like protein, was then analyzed by triple-primer RT-PCR using three primers to allow the co-amplification of WT-ER mRNA together with clone 4 variant mRNA, as described previously.[16] Typical results from IHA-consistent and IHA-inconsistent tumors are shown Figure 4. PCR products (bands of 281 bp and 249 bp) corresponding to WT-ER and ER clone 4 mRNAs were observed in all tumors. Using the Mann-Whitney rank sum test (two sided), the relative expression of clone 4 truncated variant ER mRNA to WT-ER mRNA was also found to be significantly ($P < 0.01$) higher in IHA-inconsistent tumors (median = 80.4%, SD = 18.7%) *versus* IHA-consistent tumors (median = 62.4%, SD = 14.4%; Figure 5)

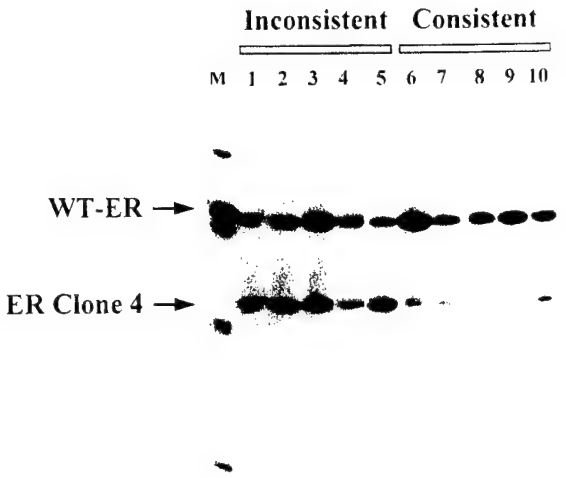

Figure 4. *Expression of clone 4 variant ER mRNA in tumors representative of IHA-inconsistent* (lanes 1 *to* 5) *and IHA-consistent* (lanes 6 *to* 10) *tumor subgroups. RNA extracted from tumors was analyzed by triple-primer PCR as described above.* Upper *and* lower arrows *show wild-type and clone 4 corresponding signals, respectively.*

Discussion

Using PCR-based approaches that allow the investigation of the prevalence of different exon-deleted and truncated ER variant mRNAs within breast tumor samples, we have investigated ER variant mRNA expression within 63 breast tumors that presented similar (IHA-consistent) or different (IHA-inconsistent) results when assessed for ER expression by IHA performed with an antibody (1D5) recognizing the amino terminal as compared with an antibody (AER311) targeting the carboxy terminal of the ER protein. We have found that, whereas variants such as D7-ER, D4-ER, D3–4/ER, and D4/7-ER are detected at the same frequency in IHA-inconsistent and IHA-consistent breast tumors, other variants, including D2–3/7-deleted, D2–3-4-ER, and D-3–7-ER, are preferentially detected in IHA-inconsistent cases. This difference between subgroups was statistically significant for two of these variants: exon-2–3-4-deleted ER and exon-3–7-deleted ER. Both of these two variant mRNAs possess sequence modifications that introduce a shift in the WT-ER coding sequence that would encode ER-like proteins containing the amino-terminal TAF-1 transactivation domain but missing all the carboxyl-terminal extremity of WT-ER protein (Figure 1). These putative variant ER proteins would therefore theoretically be recognized by 1D5 antibody but not AER311 antibody. Furthermore, detectable expression of the subset of variant mRNAs able to encode truncated ER-like proteins (except the uniformly prevalent D7-ER variant that was detected in all but 6 tumors of the 63 studied) was significantly higher in the IHA-inconsistent tumor group. In contrast, detectable expression of variants encoding in-frame proteins that should be recognized by both antibodies was no different between tumor subgroups. Taken together, these results are in keeping with the hypothesis that ER variant mRNAs encoding truncated ER proteins may participate in the synthesis of ER-like proteins differentially recognized by 1D5 and AER311 antibodies. This assumption is also further supported by the results obtained using a quantitative PCR-based approach applied to the same tumors, which indicate that IHA-inconsistent tumors also possess significantly higher levels of ER clone 4 truncated variant relative to WT-ER compared with IHA-consistent tumors.

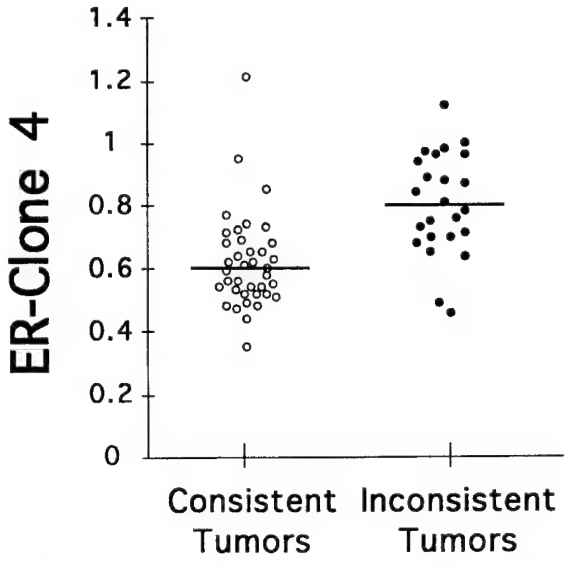

Figure 5. *Comparison of the relative expression of ER clone 4 variant mRNA in IHA-inconsistent breast tumors and in IHA-consistent breast tumors. For each sample, the mean of three independent measures of clone 4 expression was expressed as a percentage of the corresponding wild type ER signal. The difference between two groups is statistically significant (P < 0.01, Mann Whitney rank sum test, two sided).*

Until the development of antibodies that are specific for individual ER variant proteins, the premise that proteins encoded by ER variant mRNAs may directly interfere with ER immunodetection and determination of ER status by IHA remains to be proven. It is clear from *in vitro* laboratory studies that ER variants can encode proteins that possess a variety of dominant negative, positive, or undetectable activities when tested for their ability to interfere with transactivation of classical ER enhancer sequences/elements.[6–9,19] Thus, although we and others[4,5] have observed a relative increase in amino-terminal signal that may correspond to increased truncated ER proteins, the functional implications in terms of response to endocrine therapy will depend

on the nature of the specific ER variant activities in a given tumor.

Although a good correlation between ER-DCC and ER-IHA is often found, approximately 20% of cases are discordant.[5] It is believed that the cause of this discordance is multifactorial and both ER-DCC-positive/IHA-negative and ER-DCC-negative/IHA-positive cases have been attributed overall to tumor heterogeneity, sampling, variable frozen tissue handling, and formalin fixation.[20] However, an explanation for discordant results is not always apparent in specific cases.[20] Thus, although recent studies have shown that immunodetection using 1D5-IHA can accurately predict endocrine response of breast cancer,[21] the relative predictive value of ER-DCC *versus* ER-IHA is still under debate.[20–23] In the light of our results, and laboratory evidence to suggest that ER variant proteins encoded by ER variant mRNAs may participate in endocrine response,[6–14] it may be important to assess ER variant expression in future studies concerning ER-IHA status and response to endocrine therapy.

Interestingly, the two exon-deleted ER variant mRNAs, the expression of which was shown here to be correlated to inconsistent results by IHA (ie, D2–3-4-ER and D-3–7-ER), have not been detected until recently.[15] However, our previous studies[5] indicate that expression of these variants may be associated with high-grade tumors and high ER level, respectively.[15] Similarly, we have shown that a higher level of ER clone 4 mRNA expression correlates with tumor progression and poor prognosis.[16,24] This suggests not only that these ER variant mRNAs may contribute to discrepant IHA results but also that alteration of their expression is associated with tumor progression.

In conclusion, we have found a significant correlation between expression of certain ER variant mRNAs and inconsistent IHA results after assessment and comparison of ER expression with antibodies directed to either amino- or carboxyl-terminal epitopes in human breast cancer. These data add to the growing body of evidence that suggests that ER variants may be translated *in vivo* into ER-like proteins.[5,25,26] Finally, these results suggest that ER variant expression may be an important parameter to consider in the determination of ER status in human breast cancer.

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EXPRESSION OF ESTROGEN RECEPTOR-*BETA* IN HUMAN BREAST TUMORS

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ABSTRACT

The expression of a recently described novel estrogen receptor, ER–β, was detected in several human breast tumor biopsy samples and several human breast epithelial cell lines using reverse transcription and polymerase chain reaction (RT-PCR) analysis. Cloning and sequencing of the PCR product from a breast tumor confirmed the identity of the sequence with that of the ER–β mRNA previously reported in human testis. The expression of ER–β was not correlated with that of ER-α, and both ER–α positive and ER–α negative cell lines expressed ER–β mRNA. However, some breast tumors and some cell lines coexpress ER–β and ER–α mRNA. Our data support a possible role for ER–β in human breast cancer.

Estrogen signal transduction plays an important role in both normal and neoplastic mammary tissue (1). The principal mechanism by which the effects of estrogen are mediated in either normal or neoplastic target cells is via an initial interaction with the estrogen receptor (ER), a member of the steroid/thyroid/retinoid receptor gene superfamily (2). Recently, a novel ER, referred to as ER–β was cloned and characterized from human testis (3) and its rat homolog was cloned and characterized from rat prostate (4). The ER–β protein has similarities to the classical ER referred to as ER-α, in terms of structure and function. Both of these proteins have a high degree of conservation of the DNA and ligand binding domains (3), while the A/B, hinge (D) and F domains are not conserved (3,4). Transient expression assays have shown that ER–β can bind estradiol and can transactivate estrogen regulated reporter genes, although less efficiently than ER-α, and antiestrogens can inhibit this effect (3,4). Further, the tissue distribution of ER–α and ER–β although not identical appears to overlap in some cases (3,4). Therefore ER–β may be involved independently in estrogen signal transduction in some tissues but in other tissues may contribute with ER–α in estrogen signal transduction.

Estrogen has an important role in human breast cancer, however, perturbations of ER signal transduction are thought to contribute to tumor progression and the eventual development of a hormone-independent and more aggressive phenotype (5-7). The expression of ER–β in normal or neoplastic mammary tissue has not been reported, and it is important to determine if ER–β is expressed in breast cancers and therefore could potentially contribute to estrogen signal transduction in this tissue.

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Materials and Methods

Human tissues, cell lines and RNA extraction.

Forty human breast tumor specimens left-over from steroid receptor assays were obtained from the Manitoba Breast Tumor Bank. Fourteen tumors were ER negative (ER < 3 fmol/mg protein), with progesterone receptor (PR) values ranging from 0 to 19.9 fmol/mg protein (median: 11.85 fmol/mg protein). Twenty six tumors presented ER levels ranging from 4.7 to 304 fmol/mg protein (median: 33.5 fmol/mg protein) and PR levels ranging from 4.1 to 764 fmol/mg protein (median: 50.5 fmol/mg protein). Total RNA was extracted using the guanidinium thiocyanate/cesium chloride method (8) as previously described (9). The human testis sample was obtained through the Manitoba Breast Tumor Bank and the MCF 10A1, MDA MB 231, T-47D and T-47D-5 cell lines were grown as previously described (10, 11). Total RNA from the cell lines and testis sample was extracted using Trizol reagent (Gibco/BRL) according to the manufacturers instructions, and the integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (9).

RT-PCR and Primers.

Total RNA (1.5 μg per reaction), denatured at 65°C for 5 min, was reverse transcribed in a final volume of 15 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 μM random hexamers (Gibco/BRL) and 150 units M-MLV reverse transcriptase (Gibco/BRL). The reaction was allowed to proceed for 60 min at 37°C and was then terminated by heating at 90°C for 5 min.

One μl of this reaction was amplified by PCR in a final volume of 25 μl (if analyzed on 1.8% agarose gels stained with ethidium bromide) or 10 μl (if incorporating [α-^{32}P] dCTP and analyzed on 6% urea-PAGE), containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 4 ng/μl each primer and 0.02 unit/μl of Taq DNA Polymerase (Gibco/BRL).

The primers for ER–α were: ER–α upper (sense) 5' - CAG GGG TGA AGT GGG GTC TGC TG - 3' (priming site in exon 4, nucleotides 1060 - 1083 as numbered in reference 13); ER–α lower (antisense) 5'- ATG CGG AAC CGA GAT GAT GTA GC - 3' (priming site in exon 6, nucleotides 1520 - 1543). The PCR conditions were 25 cycles of 1 min 94°C, 30 sec 60°C , and 1 min 72°C, and 20 μl of the PCR reactions were electrophoresed in agarose gels (1.8%) and visualized by ethidium bromide staining.

The primers used to amplify ER–β cDNA were: ER–β upper (sense) 5'- TGC TTT GGT TTG GGT GAT TGC - 3' (nucleotides 1164 - 1185 as numbered in reference 3); ER–β lower (antisense) 5'- TTT GCT TTT ACT GTC CTC TGC - 3' (nucleotides 1402 - 1423). The PCR conditions were 1 min 94°C, 30 sec 58°C, and 30 sec 72°C, for 30 cycles. PCR was done in the presence of [α-^{32}P] dCTP (3000 Ci/mmol, 1 μCi per 10 μl reaction), and 4 μl of the reaction separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and autoradiographed. In some cases, the PCR was done in the absence of radioactivity for 40 cycles, and 20 μl of the PCR reactions were electrophoresed in agarose gels (1.8%) and visualized by ethidium bromide staining.

In order to control for errors in input of cDNA used in PCR reactions, amplification of the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel using GAPDH primers (sense 5' - ACC CAC TCC TCC ACC TTT G - 3'; antisense 5' - CTC TTG TGC TCT TGC TGG G - 3') for 25 cycles of 1 min 94°C and 30 sec 52°C. PCR products were separated on agarose gels (1.8%) and visualized by ethidium bromide staining. All PCR reactions were performed at least twice in separate experiments.

PCR products from human testis and an appropriate breast tumor thought to represent ER–β were subcloned into the cloning vector, pGEM-T Easy (Promega) as previously described (12). Double stranded mini-prep DNA from two independent clones from each tissue was sequenced using a T7 Sequencing kit (Pharmacia) following the manufacturers protocol.

Results

Forty human breast cancer biopsies were analyzed for the expression of ER–β using radioactive RT-PCR as described above. It has previously been shown that the human testis expressed ER–β mRNA at relatively high levels, and consequently RNA extracted from a sample of non-malignant human testis was used as a positive control. A 259 bp PCR product of varying intensity was detected in 70% of the breast biopsy samples analyzed (Figure 1, panel A). Several tumors displaying high, intermediate and low levels of ER–β expression using the radioactive PCR were reanalyzed using 40 cycles in a non-radioactive PCR. A 259 bp band equivalent to that found in the testis was detected in tumors displaying a strong signal in the radioactive PCR, while little if any product was detected in those tumors displaying intermediate and low signals in the radioactive PCR (Figure 1, panel B). The 259 bp signal is unlikely to result from amplification of contaminating genomic DNA, as the primers used were chosen to prime in what has been suggested to be separate exons (3). Moreover, an equivalent signal was obtained using cDNA in which the RNA had been treated with DNase I prior to reverse transcription (data not shown).

The 259 bp DNA fragments from the testis and a breast tumor sample in which a strong ER–β signal was detected were subcloned and sequenced. The tumor sequence was identical to the testis sequence, and matched that previously published for the human ER–β mRNA (3).

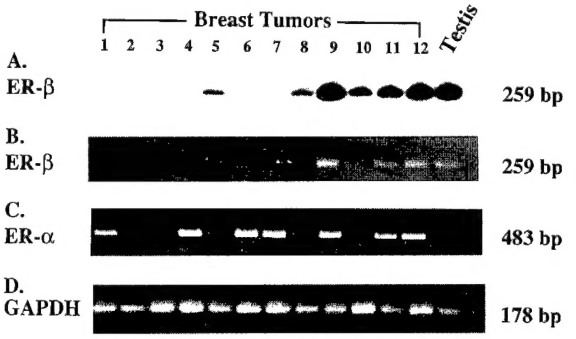

FIG. 1. **A:** Radiolabelled RT-PCR products using primers for ER–β and 30 cycles of PCR, separated on 6% PAGE containing 7M urea. Twelve human breast cancer biopsy samples and a positive control from a human testis sample are shown. The exposure was overnight with an intensifying screen. **B:** The same panel of human breast cancer biopsy samples and the human testis sample amplified with ER–β primers for 40 cycles of PCR separated on 1.8% agarose and stained with ethidium bromide. **C:** RT-PCR products using primers for ER–α from tumors and the testis sample shown in panels A and B separated on 1.8% agarose and stained with ethidium bromide. **D:** Expression of GAPDH in the tumor and testis samples shown in panels A, B and C.

Expression of ER–α mRNA in the testis and breast tumor samples was investigated using RT-PCR. The expected 483 bp DNA fragment was detected in 90% of the breast cancer biopsy samples by ethidium bromide staining (Figure 1, panel C) but no ER–α mRNA was detected in the testis sample (Figure 1, panel C). No correlation was seen between ER–α and ER–β

mRNA expression in the breast cancer biopsy samples. However, it was apparent that both genes could be expressed within the same tumor sample in some cases (see tumor samples in Figure 1, lanes 9,11,12).

To determine if the differences in level of detection were due to errors in input of cDNA used in PCR reactions, amplification of the ubiquitous GAPDH cDNA was performed in parallel using GAPDH primers (Figure 1, panel D). The results suggest similar levels of GAPDH in all samples analyzed.

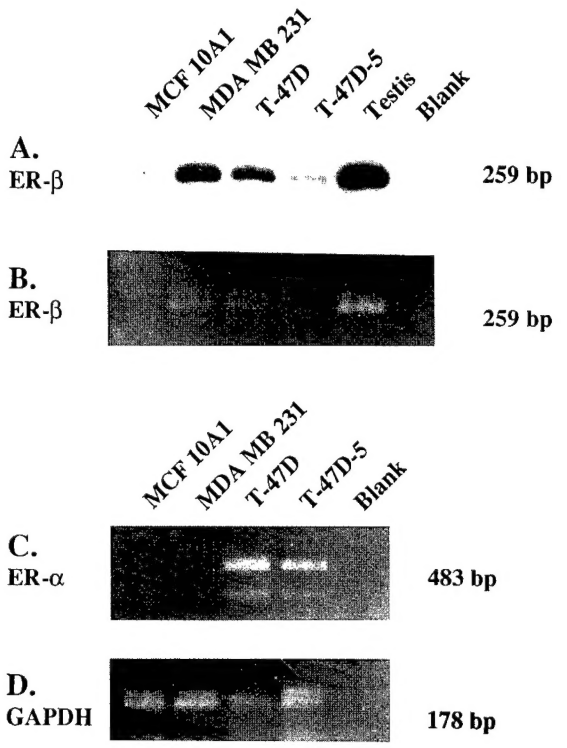

FIG. 2. **A:** Radiolabelled RT-PCR products using primers for ER–β and 30 cycles of PCR, separated on 6% PAGE containing 7M urea. Four human breast epithelial cell lines and a testis positive control are shown. The exposure was overnight with an intensifying screen **B:** The four human breast cell lines shown in panel A amplified for 40 PCR cycles using primers for ER–β separated on 1.8% agarose and stained with ethidium bromide. **C:** Expression of ER–α in the human breast cell lines shown in panels A and B using RT-PCR. **D:** Amplification of GAPDH in the four epithelial cell lines shown in panels A, B and C.

The heterogenous nature of human breast tumor samples with respect to cell type (normal and neoplastic breast epithelial cells, normal stroma, myoepithelial cells, infiltrating lymphocytes) made it difficult to determine if the ER–β mRNA was expressed exclusively in tumor cells. To address this issue we analyzed the expression of ER–β mRNA in several human breast epithelial cell lines, including breast cancer cell lines. The expected 259 bp band was detected at varying levels in all breast epithelial cell lines following autoradiography of radiolabelled RT-PCR products (Figure 2, panel A), and was detected by ethidium bromide staining of nonradioactive RT-PCR products obtained from T-47D and MDA MB 231 cells (Figure 2, panel B).

ER–β mRNA was detected in cell lines which were both ER–α positive (T-47D, T-47D-5) and ER–α negative (MDA MB 231, MCF 10A1). ER–α mRNA expression was determined by RT-PCR (Figure 2, panel C). Differences in signal are unlikely to be due to differences in input cDNA as shown by the equivalent GAPDH signal observed in all samples (Figure 2, panel D).

Discussion

The data presented in this paper provide evidence for the expression of the ER–β gene in human breast epithelial cells. Our results are the first, to our knowledge, to address the issue of ER–β expression in either normal or neoplastic breast tissue or cells. ER–β mRNA was detected in both human breast tumor biopsy samples and human breast epithelial cell lines growing in culture. The level of expression of this gene appeared to vary amongst tumor samples and between cell lines, but the expression was not correlated with the expression of ER–α mRNA. Indeed both ER–α positive (T-47D) and ER–α negative (MDA MB 231) cell lines, as determined by ligand binding assays (14) and RT-PCR analysis (Figure 2, panel C), were found to express relatively high levels of ER–β mRNA. Interestingly, the non-tumorigenic, apparently 'normal' human mammary epithelial cell line, MCF 10A1 (11), contained detectable ER–β mRNA suggesting the possibility that ER–β may be expressed in normal human mammary epithelial cells. The ER–β has been shown to have some functional similarities to the ER–α in that it can bind estradiol-17β and activate an ERE- regulated reporter gene construct, and antiestrogens can inhibit ER–β activity in these assays (3,4). However, reduced potency of estrogen activation of ER–β with respect to ER–α was noted (3), and since marked differences between these two ERs in the A/B , hinge (D) and F domains exist the assay systems previously used may not be optimal for ER–β in terms of the cell type, the promoter and possibly the ligand (3). The detection of relatively high levels of ER–β mRNA in MDA MB 231 breast cancer cells, which have previously been shown to be ER–α negative by ligand binding assays and in this paper by RT-PCR analyses, questions the

functional significance of ER–β expression in these cells at least with regard to mediating an 17β-estradiol signal. However, the lack of any clearly defined function for this protein as well as possible different ligand preferences (15) severely limits the interpretation of such data.

Although we found no correlation between ER–α and ER–β expression, some tumors and some cell lines were found to co-express these two genes. These data are consistent with previous findings (3,4) in which ER–β expression was found to have an over-lapping but non-identical tissue distribution to ER-α. While the radioactive PCR used to screen the breast tumor biopsy samples is a highly sensitive method likely to detect very low levels of expression of ER–β, several tumors presented a strong signal equivalent to that seen in the testis sample using both radioactive PCR and by ethidium bromide staining of PCR products. Our data suggest that ER–β may have a role in breast cancer cells, and this role may be expected to differ depending on the presence or absence of expression of the classical ER-α. Further, given the similarities and differences so far identified between these two gene products our results suggest that an involvement of ER–β in estrogen signal transduction or altered estrogen signal transduction in breast tissue will have to be considered.

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